THESIS

ROOT-RHIZOSPHERE INTERACTIONS AND MODIFICATIONS

Submitted by

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ABSTRACT

ROOT-RHIZOSPHERE INTERACTIONS AND MODIFICATIONS

The interactions between the plant and rhizosphere are complex, but recent research is elucidating more about a diverse array of relationships. In response to the growing demand for natural or plant produced pesticides and herbicides, a novel method for the identification of bioactive root exudates was developed utilizing the hypothesis that exudate compounds changing in relative abundance over plant development were likely bioactive. Research investigated this hypothesis on Arabidopsis grown in vitro and then in maize grown under greenhouse conditions. Four compounds were identified as bioactive, modifying plant growth, supporting this novel method of bioactive compound identification. In a second study, it was hypothesized that Plant Growth Promoting Rhizobacteria (PGPR) could be used to induce specific changes to Root System Architecture (RSA) which could impart growth benefits in specific environmental conditions. In vitro, three bacterial strains displayed the ability to modify RSA, and in a greenhouse study with nutrient deficiency, one strain was able to impart growth benefit to Arabidospsis. Both bioactive root exudates and PGPR demonstrated the potential to create desired root morphology, suggesting that root systems could be optimized to overcome environmental limitations such as drought or nutrient deficiency. Finally, a review focusing on a novel interpretation of the relationship between plants and the rhizosphere, discusses how the plant primes the rhizosphere to support and protect its offspring.

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PGPM: plant growth promoting microbes
GC-MS: gas chromatography- mass spectroscopy
RSA: root system architecture
ANOVA: analysis of variance
LSMEANS: least square means
MS: Murashige and Skoog
GABA: gamma-aminobutyric acid
rpm: rotations per minute
LR: lateral root
QTL: quantitative trait loci
AMF: arbuscular mycorrhiza fungi
VOC: volatile organic compounds
TCA cycle: tricarboxylic acid cycle
LB: Luria-Bertani
ppm: parts per million
ISR: induced systemic resistance
PGPR: plant growth promoting rhizobacteria
DI: distilled
N: Nitrogen
OTU: operational taxonomic unit
pH: percent hydrogen
IAA: indole-3-acetic acid
CK: cytokinin
GA: gibberellin
NAA: N-acetylaspartic acid

INTRODUCTION

The study of the rhizosphere is growing in interest among researchers. Many studies have attempted to gain a better understanding of the chemicals released from the roots into the rhizosphere, and to harness the benefits of plant growth promoting microbes. The following studies were conducted to gain further insight into these phenomena, the interactions within the rhizosphere, and effects on plant growth. Also included is a review addressing a multi-generational plant growth promotion imparted by microbes or root exudates.

In the first study, a unique screening process for the identification of bioactive root exudate compounds produced by the roots and excreted into the rhizosphere was developed. It was hypothesized that exudates changing in relative abundance over plant development were bioactive compounds, exuded at different levels to impact the rhizosphere in some way. Twenty two root exudate abundances were identified as significantly different at distinct developmental stages of Arabidopsis. Possible bioactive compounds were tested for their effects on the growth of Arabidopsis and maize. Results showed that some root exudates promoted growth of either roots or whole plant biomass, and some were growth suppressors. These results support the validity of the screening process developed and suggest possible root exudate uses in agriculture.

In the second study, Plant Growth Promoting Rhizobacteria (PGPR) were investigated for their potential use as targeted Root System Architecture (RSA) modifiers. Research investigated the hypothesis that PGPR could induce specific changes in RSA that could be utilized in different agricultural systems. Five PGPR were used to study RSA modifications in vitro: *Bacillus pumilus, Bacillus atropheus, Bacillus subtilis, Burkholderia* sp., *Mitsuria* sp. One strain, *Bacillus pumilus* CL29, was selected for further investigation in a nutrient deficient study to

examine whether RSA modifications could facilitate increased nutrient uptake. The results from these studies indicate potential for optimized RSA modifications induced by bacteria to help plants overcome nutrient limiting conditions.

Finally, the review paper herein discusses evidence of a multi-generational relationship between the plant and the rhizosphere, a relationship the authors have termed “soil memory”. We propose that plants attempt to increase their fecundity though a strong association with the abiotic soil components and, more importantly, the soil microbiome. Although there are many questions surrounding the mechanisms of this phenomenon, there is increasing evidence of its existence. We review observations and mechanisms related to soil memory, and report means to utilize our understanding for sustainable agriculture. The studies conducted for this thesis could be included as more evidence supporting this hypothesis of soil memory, and the mechanisms plants use surrounding it, a connection which is addressed further in the conclusion.

CHAPTER 1 MODIFICATION OF PLANT GROWTH BY ROOT EXUDATE APPLICATION[1]

Synopsis

Roots produce numerous exudates in the rhizosphere, but the bioactivity of these compounds has been hard to asses due to the methodological inadequacies. In the present study, we developed a unique screening process for identification of bioactive compounds secreted by roots, and evaluate selected compounds for bioactivity in plants. First, we measured relative abundances of root exudates produced during four Arabidopsis growth periods using GC-MS. Then, we selected root exudate metabolites which were produced in significantly different ($\alpha \leq 0.05$) relative abundances at distinct growth stages with the hypothesis that these compounds were bioactive, and would induce changes in the rhizosphere. We tested those possible bioactive compounds for effects on growth of Arabidopsis and maize plants by analyzing root growth in vitro and later in greenhouse pot experiments. The results suggest that some root exudate compounds are stimulants of above ground biomass or root growth in Arabidopsis, while others may be universal root growth stimulators, or act as root growth inhibitors in maize. Adipic acid significantly decreased biomass but increased root length in Arabidopsis in vitro, and decreased root volume in maize. Levoglucosan increased root length significantly in Arabidopsis and by 9% in maize (non-significant) in maize, and N-acetylaspartic acid significantly decreased Arabidopsis biomass. These results support that specific bioactive root compounds could be

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identified using this novel screening methodology, and then applied to induce desirable root architecture in specific agricultural systems.

Introduction

Plants produce bioactive compounds which have been utilized in pharmaceutical development and as food additives, as well as for pesticides and in other agricultural applications (Azmir et al., 2013). In the environment, the action of plant bioactive compounds on other organisms is concentration dependent; secondary metabolites such as phenolics, alkaloids jasmonates, amino acids etc. may inhibit or promote plant growth depending on concentration (Farooq et al., 2013; Baetz and Martinoia, 2014). Natural plant products like root exudates are generally more environmentally friendly, making them an attractive candidate for use as herbicide, pesticides, (Uddin et al., 2014) or in other agricultural applications. Still, the investigation of root exudates has been undervalued historically (Baetz and Martinoia, 2014) and only about 15% of higher plants have undergone thorough investigation of their phytochemicals (Cragg and Newman, 2013). The potential for the existence of unknown bioactive compounds actuates development of an investigative method for identification of biologically active compounds.

One methodology aimed at bioactive compound identification, the ethnopharmacological approach, utilizes historic knowledge of medicinal plant parts and biological assays followed by activity-oriented separation to identify novel chemical entities with pharmaceutical application potential (Brusotti et al., 2014). Application oriented approaches, however, are cumbersome; requiring many screenings and separations. Methods for extraction of bioactive compounds from plant tissue depend on an understanding of the chemistry of the bioactive compound (Azmir et al., 2013) limiting the search for novel compounds. Methods which require prior knowledge of

plant compound bioactivity further restrict the search for identification of novel uses for previously identified compounds. To avoid tissue extraction complications, root exudate compounds are optimal candidates for bioactivity identification. However, because of the vast range of mixtures secreted into the soil (Badri and Vivanco, 2009), evaluating every exudate is unfeasible. Therefore, a new methodology is needed to identify bioactive compounds that could circumnavigate the shortcomings of other approaches.

Previous research has demonstrated that classes of root exudates (sugars, amino acids, sugar alcohols, and phenolics) vary in excretion levels at different Arabidopsis developmental time points, thought to depend on developmentally controlled genetic coding (Chaparro et al., 2013). Early in development, Arabidopsis plants exude many sugars. Later, during flowering, Arabidopsis produces secondary metabolites possibly related to defense and stress resistance in order to select a more beneficial rhizomicrobiome (Chaparro et al., 2013; Chaparro, Badri, and Vivanco, 2014). Subsequently, the bioactivity of these root exudate groups was investigated in the rhizosphere. The results suggest that the differentially produced root exudates dictated the microbiome composition as well as its functions- selecting for microbes which fix nitrogen or microbes which synthesize steptomycin; theoretically to fulfill the needs of the plant at that developmental time point (Chaparro, Badri, and Vivanco, 2014). Based on these findings, we developed a new methodology for identification of single bioactive compounds using the rationale that differently regulated and therefore non-constitutively produced chemicals are likely exuded at different levels for some purpose. Rather than grouping root exudates into chemical classes, mean relative abundance of individual exudates changing over time during Arabidopsis development were studied further. Specifically, a single exudate with significant changes in relative abundance during the two leaf growth stage (10 days), the five leaf rosette stage (17

days), the bolting stage (24 days), or the flowering stage (31 days) (Chaparro, Badri, and Vivanco, 2013) was then investigated further for bioactivity.

The purpose of this research was to develop a method for bioactive compound identification. We focused our research on root exudates with bioactivity in the model plant, Arabidopsis (the plant that the exudates were originally collected and identified from). We then tested identified bioactive compounds for agricultural use on maize. Exudates identified and selected for further investigation were non-constitutively produced; they were found to be differing in relative abundance during specific developmental time points at the 1% or 5% confidence level, with their highest expression in the final developmental stage (flowering). The question addressed in this study investigated if this method of screening root exudates could lead to the discovery of bioactive compounds. We hypothesized that the compounds changing at significant levels over development were bioactive and their application to plants would produce observable changes in growth. Analysis of bioactivity, therefore, took the form of two additional questions: (1) what are the effects of select root exudates on Arabidopsis Root System Architecture (RSA) and whole plant biomass (model organism), and (2) what are the effects of select root exudates on RSA and whole plant biomass of maize (agricultural crop)?

Methods/Materials

Root exudate identification and statistical analysis of root exudate components which change over time

Root exudates of wild type *Arabidopsis thaliana* (Col-0) were collected using the protocol previously described by (Chaparro et al., 2013), and exudates were identified using Gas Chromatography-Mass Spectrometry (GC-MS) (Chaparro et al., 2013). Briefly, 7-day old Arabidopsis plantlets were transferred to 6-well plates containing 5mL of liquid Murashige and

Skoog (MS) media (Murashige and Skoog, 1962) supplemented with sucrose (1%) and placed on an orbital shaker at 90 rpm under a 16-hr photoperiod with white fluorescent lights (45 µmol m^{-2} s^{-1}) at room temperature. When the plants reached the developmental stage of interest as defined by number of days old: the two leaf growth stage (10 days), the five leaf rosette stage (17 days), the bolting stage (24 days), and the flowering stage (31 days) (Chaparro, Badri, and Vivanco, 2013) they were gently washed with sterile distilled (DI) water to remove loosely adhering exudates and the plantlets were transferred to 6 well plates containing 5mL of sterile DI water, and returned to the orbital shaker for three days. After three days, the exudates were collected from the DI water using nylon filters of pore size 0.45 µm (Millipore) to remove root cells. Exudates were collected in three replicates at each stage of development. Filtered root exudates were freeze-dried, dissolved in sterile water, and sent to the Genome Center Core Services at the University of California Davis for GC-MS analysis. Exudate solutions were dried under nitrogen gas and subsequently underwent methoximation and trimethylsilylation derivatization. Samples were run on an Agilent 6890 gas chromatograph (Santa Clara, CA) containing a 10 m long empty guard column and stored using Leco Chroma TOF software Version 2.32 (St. Joseph, MI). Exudates were assigned by the BinBase identifier numbers utilizing retention index and mass spectrum as pertinent identification criteria (see Data Dictonary Fiehn Laboratory 2013 for more information).

Relative abundance of root exudate compounds were calculated based on total peak area of the chromatogram for all compounds including unknowns to normalize each compound between samples. To identify and quantify significant changes in mean expression levels of compounds over the four Arabidopsis developmental periods, R statistical software (Team, 2015) was used to perform Analysis of Variance (ANOVA) on each known compound to

identify significant production differences across plant development stages. Due to the large number of hypothesis tests performed, a Bonferroni family-wise error rate (or false discovery rate) correction was used to ensure correct probabilistic interpretations, producing extremely conservative estimations and significance.

Arabidopsis In vitro experiment

Wild type *Arabidopsis thaliana* (Col-0) seeds were surface sterilized using a 10% bleach solution followed by 4 rinses with sterilized Millipore water. Seeds were sown in low density for germination on MS (Murashige and Skoog, 1962) media (1% sucrose) in square plates. After seven days, seedlings were transferred aseptically in a Laminar Flow Hood to 6-well plates containing 4mL liquid media containing 100% MS. 6-well plates containing sterile liquid media were used to eliminate potential influence of microbial exudation, nutrient location, soil conditions, and pot/environment interactions. Treatments consisting of single root exudates were added to each well in 100 nM concentrations based on previous investigation of exudate concentrations in the soil (Badri et al., 2013). For combination treatments, each compound was incorporated at 100nM concentrations. The compounds selected and investigated were: gamma-aminobutyric acid (GABA), levoglucosan, malic acid, maleic acid, galactanol, N-acetylaspartic acid, adipic acid, glutamic acid, fumaric acid, and a combination treatment containing maleic acid, malic acid, and fumaric acid. Wells containing MS without additional compound served as a control. Plates were placed on a rotary shaker (82 rpm) at room temperature under white lights (32W T8) and a 16hr photoperiod. The rotary shaker maintained mixing in an effort to sustain uniform distribution of MS nutrients as well as prevent hypoxia. After ten days, before plants grew too large and started interacting the with 6-well plate wells, roots of half of the plants were scanned using winRHIZO™ root-scanning imaging software (Regent Instruments Inc., Ottawa,

Canada) to quantify root architecture. The remaining plants were oven-dried for three days and weighed to assess whole plant dry biomass. Plants that showed signs of bacterial or fungal infection were excluded, resulting in approximately equal sample sizes for each treatment. A total of 266 plants were evaluated for RSA (19-27 plants corresponding to each treatment and the control) and 279 plants for biomass (21-27 plants corresponding to each treatment and control).

Maize Greenhouse experiments

Maize experiments were conducted in greenhouse conditions to identify compounds with potential for agricultural application. Twenty maize seeds (Dekalb® Hybrid Corn Seed Blend DKC43-48RIB) were submerged in 100nM concentrations of one of three compounds, adipic acid, levoglucosan, and malic acid, and placed on an orbital shaker for 2 hours to soak (control maize seeds were soaked in sterile DI water). Treatments adipic acid and levoglucosan were selected due to their bioactivity in Arabidopsis, while malic acid was selected because it was not. This methodology was used to identify the minimal number of applications needed to produce significant results. Seeds were planted at 1inch depth in Deepots (Ray Leach "Cone-tainer"™ SC10) filled with Turface (Athletics™, Profile Products, Buffalo Grove, IL) and misted for 35 seconds every 30 minutes during the 16 hour photoperiod. Each plant was fertilized with 10mL of 50% Hoaglands solution with Fe_2HO_4 (Phytotech Lab, Shawnee Mission, KS) every three days. Three weeks after germination, before plant roots began interacting with the pot, all plants were harvested and roots were scanned using winRHIZO™ root-scanning software. Immediately following scanning, whole plants were oven dried for three days and then weighed for dry biomass. Twenty plants were evaluated in each treatment as well as twenty control plants.

Statistical analysis of growth responses

The RSA, biomass, and growth data sets for both Arabidopsis and maize were visualized in R software (Team, 2015)(version 3.1.3 (2015-03-09) -- "Smooth Sidewalk" © 2015), and outliers more than three standard deviations away from the population mean estimate were removed. Following outlier removal, Analysis of Variance (ANOVA) tests were performed for each of the RSA variables: root length (cm), root surface area (cm^2), root volume (cm^3), and average root diameter (cm), and for biomass (g). If assumptions of ANOVA were not met, the data was transformed for normality and equal variance. Because experiments were conducted in blocks, ANOVA was used to screen for blocking effects. If blocks influenced response variables, then a more complex model to account for blocking effects was used in subsequent analyses. If no relationship between blocks and response variables was identified, then a simplified model without accounting for blocking was used. If a significant difference ($\alpha \leq 0.1$) between treatments was identified based on ANOVA results, then the difference between each treatment and the control was estimated using Dunnett's Analysis. Estimates of central tendency (mean, median) and estimates of variability (standard deviation) were also made using Dunnett's adjustment.

Results

Root exudate identification and statistical analysis of root exudate components which changed over time

A total of 483 exudate compounds were found using GC-MS (supplemental table 1). 179 compounds were identified and underwent statistical analysis to identify significant relative abundance differences over Arabidopsis development. Twenty-two root exudate compounds were identified as significantly changing in mean relative abundance at the 5% level at one of the four developmental stages (Table 1). All compounds which were identified as significantly

changing had the highest mean relative abundance levels in the flowering stage of Arabidopsis development. Of these, N-acetylaspartic acid (NAA), levoglucosan, guanosine, and adipic acid were identified as having the lowest mean relative abundance in the first stage of development (two leaf stage) (Table 1). All other significantly changing compounds had the lowest mean abundance level in the second stage of development, the five Leaf Rosette stage (Table 1). The highest and lowest relative abundance differed by orders of magnitude. The exudates used for further experiments were: GABA, levoglucosan, maleic acid, galactanol, N-acetylaspartic acid, glutamic acid, adipic acid, malic acid, and fumaric acid based on immediate availability.

Table 1. Mean relative abundance of root exudate compounds over four developmental stages of Arabidopsis (Two Leaf; days, 5 Leaf Rosette; days, Bolting; days, and Flowering; days) found to be significantly changing at the 5% significance level ($\alpha \leq 0.05$). The lowest mean abundance is highlighted in orange, the highest mean abundance is highlighted in green. Significant difference points are unique to each compounds.

Compound	Two Leaf	5 Leaf Rosette	Bolting	Flowering
Suberyl Glycine	1,876.0	131.7	196,686.7	508,497.3
Raffinose	129.3	113.0	285.7	1,147.0
N-acetylaspartic Acid	94.7	101.7	285.7	587.0
Maleic Acid	1,665.3	749.3	11,363.7	21,257.0
Levoglucosan	166.0	169.7	461.0	789.0
Inulobiose	283.3	110.7	16,647.3	28,349.7
Guanosine	69.0	86.0	679.7	1,058.0
Glycerol-3-galactoside	8,189.0	766.3	327,435.7	455,191.7
Glutamine	91,583.3	8,038.3	495,571.3	1,006,709.7
Glutamic Acid	27,837.3	1,055.0	443,097.3	908,796.0
Galactinol	7,952.7	1,217.7	129,744.7	239,234.3
GABA	44,071.0	2,723.0	684,531.7	954,341.0
Xylonolactone NIST	511.7	185.3	3,853.7	15,728.3
Parabanic Acid NIST	732.7	346.0	5,548.0	19,612.0
N-acetylmannosamine	1,734.3	123.0	9,850.0	30,913.0
N-acetyl-D-hexosamine	2,581.3	296.0	8,459.0	25,593.7
Malic Acid	24,372.7	539.0	149,607.3	340,263.3
Myo-inositol	5,668.3	401.7	36,903.0	108,351.0
Fumaric Acid	6,787.0	1,568.3	124,750.7	284,439.3
Dihydoxyacetone	95.7	64.3	334.0	366.3
Aspartic Acid	22,713.0	1,263.7	259,026.3	597,693.3
Adipic Acid	422.7	484.0	765.7	1,022.0

In Vitro Arabidopsis Biomass

Treatment of Arabidopsis in vitro found four compounds resulted in significant ($\alpha \leq 0.1$) changes in Arabidopsis biomass compared to control (mean=0.009g, sd=0.00353). Fumaric acid (mean=0.0097g, sd=0.0026) significantly increased plant biomass (p=0.04138). Alternatively, adipic acid (mean=0.0089g, sd=0.0023) (p=0.00816), levoglucosan (mean=0.0872g, sd=0.0032) (p=0.02069), and N-acetylaspartic acid (mean=0.00898g, sd=0.0025) (p=0.01257) treatments resulted in significantly decreased biomass (Figure 1, Table 2).

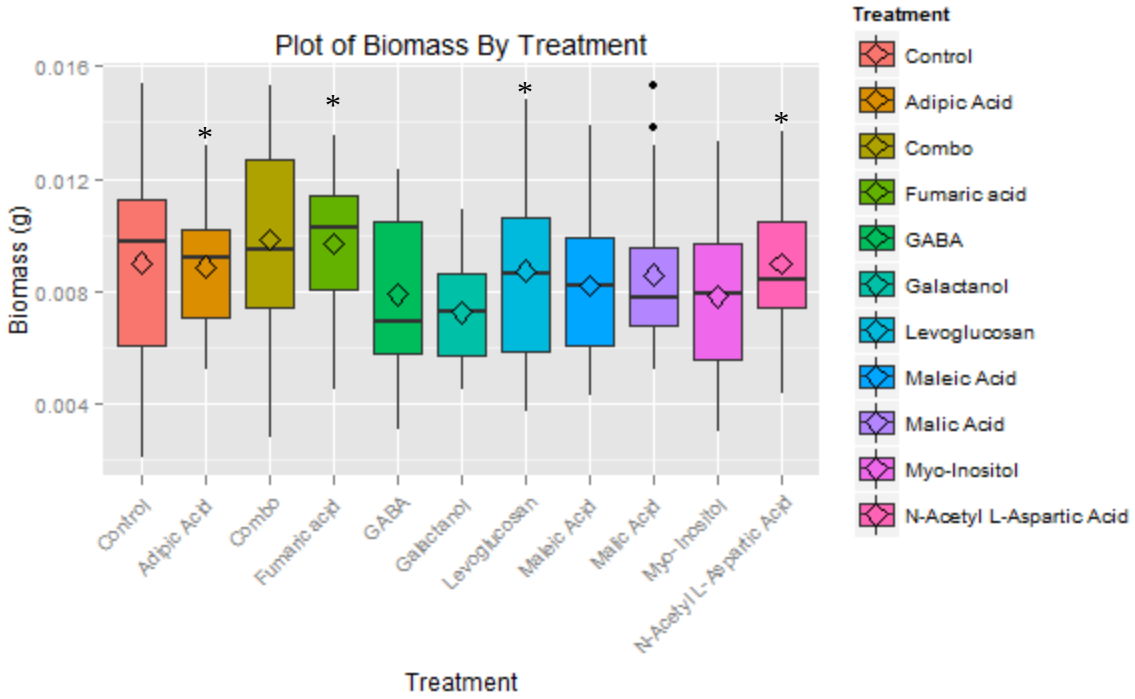

Figure 1. In vitro Arabidopsis dry weight biomass (g) by treatment. From 21-27 plants corresponding to each treatment and control were assessed for dry weight for a total of 273 plants. Fumaric acid increased biomass while adipic acid, levoglucosan, and NAA decreased biomass significantly ($\alpha \leq 0.1$).

In Vitro Arabidopsis RSA

Root exudate treatment of Arabidopsis resulted in significantly increased root length compared to control (mean=105.07 cm, sd=28.99) for treatments: adipic acid (mean=124.18 cm, sd=22.11904) (p=0.0109) and levoglucosan (mean=121.56 cm, sd=22.64) (p=0.0656) at the

α≤0.1 level (Table 2, SFigure 1). Root exudate treatment resulted in no significant changes in root surface area, average root diameter or root volume with different treatments.

Greenhouse Experiments: Maize

Biomass of maize did not significantly change with treatment. No significant differences between control and treatments were identified in root length, although the estimated mean for root length in maize exposed to levoglucosan was 127.67cm longer (mean=1548.42cm sd=228.27) than the average root length of the control (mean=1420.64 cm, sd=257.6) (Figure 2). Root surface area and average root diameter of maize was not found to be significantly different between treatments and control. There was a significant (α≤0.1) decrease in root volume for maize treated with adipic acid (mean=2.2 cm^3, sd=0.37) compared to control (mean=2.73 cm^3, sd=0.99) (Table 2, SFigure 2).

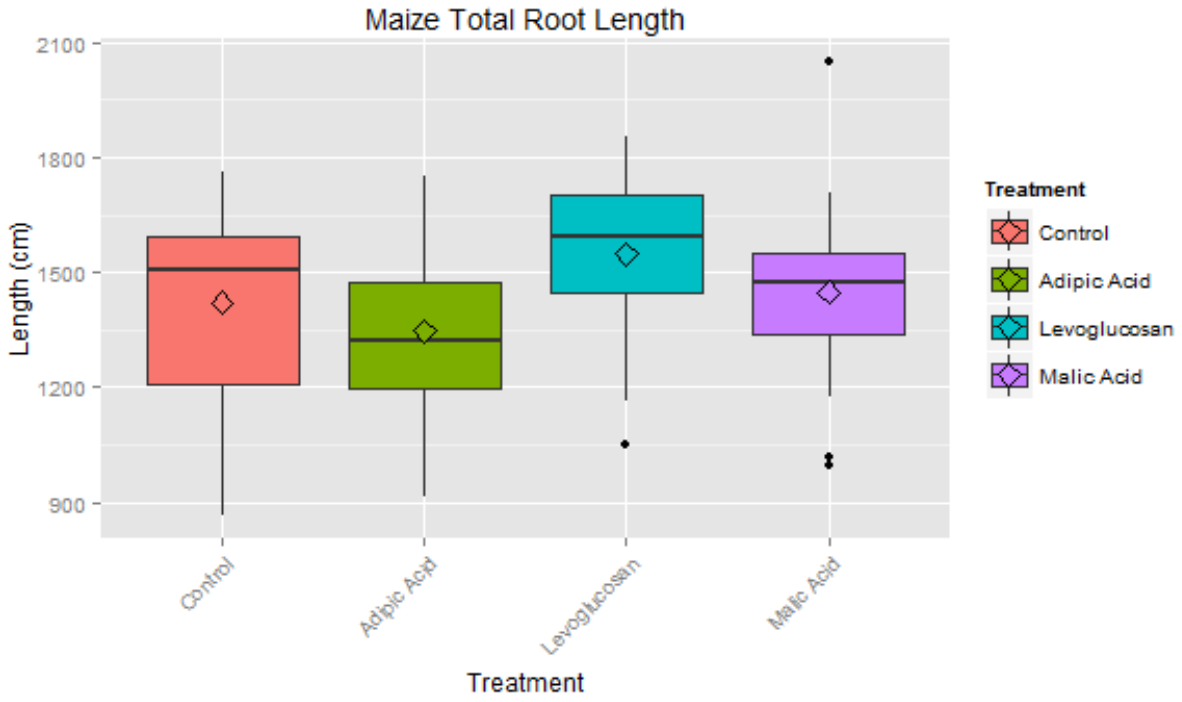

Figure 2. Total root length of Maize in greenhouse experiments. Twenty seedlings were evaluated from each treatment and the control. No significant changes in total root length of maize identified.

Table 2. Summary table of exudate effects on biomass and RSA in Arabidopsis and maize

Compound	Arabidopsis		Maize
Fumaric Acid	Increased biomass (g)		
Adipic Acid	Decreased biomass (g)	Increased root length (cm)	Decreased root volume (cm^3)
Levoglucosan	Decreased biomass (g)	Increased root length (cm)	9% Increase in root length (cm) (non-significant)
NAA	Decreased biomass (g)		
Malic Acid	No significant effects		No significant effects

Discussion

Bioactive compounds have been isolated from various plant parts including leaves, stem, flowers, and fruits (Azmir et al., 2013), while discovery of bioactive root exudates appears less often in the literature overall. Recently, however, the investigation of bioactive root exudate compounds has indicated that they play a large role in modifying rhizosphere composition (Chaparro, Badri, and Vivanco, 2014), for example by acting as attractants, stimulants, and signaling molecules (Baetz and Martinoia, 2014). Used in food products, bioactive root exudate investigation has led to the discovery of many antibacterial and antifungal compounds (Baetz and Martinoia, 2014) that could be used in food preservation (Shan et al., 2008). Used in agriculture, natural plant products with bioactivity are a more environmentally friendly alternative to synthetic chemicals (Uddin et al., 2014).

Multiple experiments have been conducted to test the effects of bulk root exudates on neighboring plants of either the same or different species (Semchenko, Saar, and Lepik, 2014), however, few studies have investigated the effects of single root exudate components. Despite limited understanding of mechanisms, multiple root derived compounds have been applied successfully in preliminary agricultural experiments. For example, formulated sorgoleone, a sorghum root exudate, suppressed both germination and shoot growth of weeds (Uddin et al.,

2014). Sorghum has been studied as an allelopathic crop for many years, and has been found to improve growth in some crops by suppressing competitors (Farooq et al., 2013a), so the exploration of its root exudates for bioactivity was primarily based on prior knowledge. Another fruitful approach to bioactive root exudate identification was developed from investigation of a constitutively produced terpene rhizathalene A, which was found to be involved in defense against belowground herbivory (Vaughan et al., 2013). Rather than relying on anecdotal information as a starting point, the present study identified potentially bioactive compounds by evaluating changes in their relative abundances during development, using highly conservative statistical analysis (Bonferroni). Analysis of the bioactivity of these select compounds was pursued through investigating application to the model plant, Arabidopsis, as well as an agro-economically valuable crop, maize.

Our initial screening process of eliminating constitutively produced root exudate compounds identified twenty-two compounds with significant changes in relative abundance. Some of these compounds have already been identified as bioactive, supporting the validity of the screening. GABA, for example, has been studied as a signaling molecule in both animals and plants; accumulating in plant tissue in response to stress, and regulating plant growth (Ramesh et al., 2015). GABA also produces significant changes in the soil microbial community, being positively correlated with certain operational taxonomic units (OTUs), and negatively correlated with others (Badri et al., 2013). Efflux of organic anions such as fumaric acid, N-acetylaspartic acid, and adipic acid, has been associated with changes in root-cell metabolism leading to increased organic acid synthesis. Additionally, organic anions can be directly or indirectly involved in metabolic processes such as carbon and nitrogen assimilation, pH regulation, charge balance during cation uptake, and supplying bacteria with energy (see (Ryan, Delhaize, and

Jones, 2001) for review). These compounds have not, however, been applied to plants to investigate RSA modification or plant biomass manipulation, making this bioactivity a novel discovery.

Although this study only investigated one concentration of isolated exudates (100 nM), it is possible that changes in concentrations of exudates or oscillating relative abundance of exudates are responsible for eliciting different plant responses (Gruntman and Novoplansky, 2004). The exudates investigated in this experiment were produced at their highest levels in the latest developmental stage of Arabidopsis growth (flowering), suggesting that the plant may be readying the environment for the germination of its progeny. The plants may accomplish this with root exudates that decrease germination or vigor of competitors (Evidente et al., 2007; Wang et al., 2015) giving their own progeny a competitive growth advantage. Studies in maize were intentionally non-intensive, but more significant results could be identified by repeated application of the root exudate compounds, which theoretically could be accomplished through fertigation.

Our findings suggest that we have identified a few bioactive compounds with action in modifying plant growth patterns, specifically biomass or RSA. Levoglucosan significantly increased root length in Arabidopsis, as well as in maize (non-significant trend). This suggests that levoglucosan may be a universal bioactive compound responsible for signaling increased root growth. More root development leads to competitive nutrient acquisition (Ho et al., 2005; Semchenko, Saar, and Lepik, 2014) therefor levoglucosan could benefit plants grown in nutrient limiting conditions by increasing soil exploration. Fumaric acid increased Arabidopsis biomass suggesting that it may be a bioactive compound responsible for promoting above ground biomass rather than competitive root growth. This could also be adventageous in agricultural applications

if soil nutrient levels were not limiting but that above ground biomass was desirable such as aquaponics. Adipic acid significantly decreased the root volume in maize seedlings, while the same exudate increased root length in Arabidopsis demonstrating that these exudates should be investigated on more crop species to identify variable plant growth responses. Malic acid was not identified as bioactive in Arabidopsis, and subsequent experiments found it was also not bioactive in maize. These findings, however, do not eliminate the possibility of malic acid bioactivity in other plants, or in other interactions.

Despite identifying twenty two root exudate compounds changing significantly over the development of Arabidopsis, only eight individual compounds were investigated for growth effects in this study. The remainder of the exudate compounds, or compounds from other plants found to be changing significantly over the plant's development, should be investigated for bioactivity in Arabidopsis as well as other plants. Additional investigation of these isolated exudates needs to be conducted for their effects on the rhizosphere community. Furthermore, experiments with variable concentrations of exudates should be conducted to further support the hypothesis that plant responses to root exudate compounds are concentration dependent (Gruntman and Novoplansky, 2004).

Conclusions

Twenty two root exudates changed significantly in mean abundance levels over Arabidopsis development (Table 1). Of these exudates, four were identified as bioactive in plants, significantly manipulating RSA or biomass, indicating the validity of the unique screening process developed here. These bioactive exudate compounds and their activity in plants are: fumaric acid, which significantly increased Arabidopsis biomass in vitro; adipic acid, which significantly decreased biomass and increased root length in Arabidopsis in vitro and

significantly decreased maize root volume in the greenhouse; levoglucosan which significantly decreased Arabidopsis biomass in vitro and increased root length; and N-acetylaspartic acid which significantly decreased Arabidopsis biomass in vitro.

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CHAPTER 2 MODIFICATION OF PLANT GROWTH BY PLANT GROWTH PROMOTING RHIZOBACTERIA[1]

Synopsis

The use of Plant Growth Promoting Rhizobacteria (PGPR) is a magnetizing topic in agricultural science in light of increasing interest in productivity and diminishing inputs of synthetic pesticides and fertilizers. In this study, we investigated the ability of five PGPR to modify root system architecture (RSA) with the purpose of investigating agricultural applications. In an in vitro experiment, we identified three bacterial strains which significantly modified RSA in Arabidopsis. In vitro, *Mitsuira* sp. strain ADR17 significantly increased the number of lateral roots, total root length, root surface area, average root diameter, and root volume. *Burkholderia* sp. strain ADR10 significantly increased lateral root branching but did not significantly increase any other root parameter. *Bacillus pumilus* strain CL29 significantly increased total root length, root volume, and root surface area, and in a subsequent growth chamber experiment, above ground dry weight (biomass) under nutrient limited conditions. These results suggest that specific bacterial strains could be used to optimize RSA to match environmental conditions or nutrient deficiencies and potentially increasing resource acquisition and assimilation in plants.

Introduction

A number of plant growth promoting rhizobacteria (PGPR) have been formulated for application in agriculture, horticulture, forestry, environmental restoration, and other scenarios to

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improve plant growth (Lucy, Reed, and Glick, 2004). PGPR can impart many different growth benefits to the plant including disease resistance, increased germination rates, root growth, yield, nutrient content, as well as increased resistance to abiotic or biotic stress (Lucy, Reed, and Glick, 2004). In return, the plant releases enormous amounts of chemicals through their roots to support these mutualistic relationships and to deter pathogenic microorganisms (Badri et al., 2009). Chemical dialogues between the roots and the rhizosphere microbes are diverse and dynamic by nature, and there are undoubtedly a great number of interactions to be elucidated.

Plant roots are critical for survival because of their role in anchorage, water and nutrient acquisition, and mediating interactions with soil microorganisms (Ristova et al., 2013). The dynamic spatial configuration of the below-ground plant body, known as Root System Architecture (RSA), is a plant's version of motility, primarily responsible for efficient resource acquisition, leading some researchers to investigate presumed optimal root system architecture to maximize plant productivity (Ristova et al., 2013; Kong et al., 2014; Rogers and Benfey, 2015). Optimal RSA needs to be specifically tailored to the shortcomings of the environment (Rogers and Benfey, 2015; Tron et al., 2015), with different root configurations for each scenario. Research indicates that plants with low lateral root (LR) branching density combined with longer LRs increase soil exploration while decreasing root competition (Kong et al., 2014). In addition, this combination of RSA features will increase resource acquisition from deeper in the soil profile where water, sulfate, nitrogen, and other soluble nutrients are located (Kong et al., 2014). In contrast, for immobile resources such as phosphorus, potassium, iron, and manganese, nutrient acquisition is best attained by high LR density and shallower, shorter root systems (Kong et al., 2014). RSA is not only controlled genetically, but is extremely dependent on environmental cues (Kong et al., 2014; Rogers and Benfey, 2015) making RSA difficult to control. Furthermore,

though many gene loci have been identified as controlling RSA, few have been successfully exploited to increase crop production (Rogers and Benfey, 2015).

Rather than attempting to modify RSA using genetic manipulation, which would require advanced knowledge of the Quantitative Trait Loci (QTLs) responsible, and a separate line tailored to each soil problem, application of a modifying agent to the crop or soil could be a viable alternative. Some studies have demonstrated changes in root architecture as a result of inoculation with arbuscular mycorrhiza fungi (AMF) or rhizobia bacteria (Li, Zeng, and Liao, 2015). For example, it is well known that the formation of root nodules by rhizobia begins with a chemical dialog followed by curling of the root hairs around the bacterial colony (Li, Zeng, and Liao, 2015). Root architecture modifications from AMF or rhizobia may involve root growth promotion and increased length and number of LR, or in different species such as soybeans, rhizobia and AMF may result in inhibited root growth (Li, Zeng, and Liao, 2015) demonstrating a host specific response. Furthermore, multiple studies have displayed the ability of bacterial volatile organic compounds (VOC) to modify RSA, specifically, *Bacillus cereus* and *Bacillus simplex,* (Gutierrez-Luna et al., 2010). Another study identified five other bacterial groups which increased total plant biomass and root development, with *Bacillus subtilis* GB03 producing the most significant results (Delaplace et al., 2015). Growth promoting bacteria able to colonize the rhizosphere of agricultural plants, and persist over multiple years, could potentially serve as an important tool in environmentally friendly and sustainable agriculture utilizing RSA re-arrangement (Vassilev et al., 2015).

The identification of novel bacteria with agricultural application from soil samples requires laborious screening processes to isolate even a few microbes for further study. Often, soil believed to have high microbial diversity is utilized as the source for novel microbe

isolation. Soil from the Peruvian rainforest of Tambopata, for example, supports a wide range of plants (Huang et al., 2013) suggesting that it may also house a diverse microbial consortia (Lagunas, Schaefer, and Gifford, 2015). The soils in the Tambopata rainforest are also relatively nutrient-poor, and the microbes are hypothesized to support plant life through supporting rapid organic matter turnover (Huang et al., 2013) or other means. Microbial exploration of this soil has resulted in the identification of two bacteria with lignin-degrading activity, *Bacillus atrophaeus* strain LSSC3 and *B. pumilus* CL29. Further investigation identified plant growth promoting activity in three strains from the same soil, *B. subtilis* T2, *B. atrophaeus* LSSC3, and *B. pumilus* CL29 (Huang et al., 2015). This plant growth promotion, however, was identified using above ground, fresh weight specimens; a proven unreliable method of evaluation (Bashan and de-Bashan, 2005). Furthermore, root growth reacts to changing environmental factors more rapidly than aboveground biomass (Muhlich et al., 2008), and is a more likely target for soil microbes. For these reasons, further investigation into the growth promotion ability of these microbes is warranted.

This study focused on the influence of selected microbes, *Bacillus subtilis* T2, *Bacillus atrophaeus* LSSC3, and *Bacillus pumilus* CL29 isolated from the rainforest soil (Huang et al., 2013) and *Burkholderia* sp. ADR 10 and *Mitsuaria* sp. ADR17 isolated from the Arabidopsis rhizosphere soil (Zolla et al., 2013), on RSA of Arabidopsis growth in vitro. The purpose of this investigation was to address whether these microbes increase root growth and resource acquisition potential belowground by means of RSA modification. The hypothesis was that microbes could create desirable RSA configurations to give the plant a growth advantage in limiting environments. Following confirmed promotion of root growth *in vitro,* a single PGPR (*Bacillus pumilus)* underwent further investigation, on Arabidopsis grown in a growth chamber

under nutrient deficient conditions.This second part of this study investigated whether *B. pumilus* promotes root growth for increased nutrient acquisition ability and therefore above ground biomass under nutrient deficient conditions.

Materials and Methods

In vitro RSA modification

Arabidopsis seeds were surface sterilized (20% v/v bleach for 1 min followed by five washes with sterilized distilled water) and placed on germination media (100% MS, 1% sucrose, 2% agar) (Murashige and Skoog, 1962) in square plates for four days in a 24±3ºC growth chamber with a 16 hr photoperiod. Three days following seed germination initiation, 3mL liquid cultures of *Bacillus subtilis, Bacillus atrophaeus, Bacillus pumilus, Burkholderia* sp., and *Mitsuria* sp. were initiated in full strength liquid Luria-Bertani broth (LB broth). Seedlings were then transferred to experimental plates: round 10 cm agar plates with 20% MS, 0.5% sucrose, and 1% agar. Two seedlings were placed along the top of the plate (allowing room for root growth without interfering with the plate), approximately 2 cm apart. Bacteria liquid cultures were centrifuged to pellet and re-suspended in phosphate-buffered saline (PBS) to a concentration of 10^6 cells/mL. Ten µL of bacterial suspension was plated on the edge opposite to each seedling (Figure 1), and aluminum foil was wrapped around the plate base to limit root exposure to light. Control plantlets were inoculated with ten µL PBS. Each seedling and subsequent plantlet served as a separate replicate. After ten days on experimental plates, plantlet roots were measured using winRHIZO imaging software (Regent Instruments Inc., Ottawa, Canada) and lateral root numbers were counted to avoid over-counting and increase accuracy. Plantlets were not removed from plates to eliminate risk of damaging the roots. Plates were discarded prior to data collection if they were contaminated with foreign microbial growth and

plantlets were excluded if they did not have a single tap root, resulting in different group sizes. 24 plantlets were measured for *B. atrophaeus,* 17 for *B. pumilus,* 11 for *B. subtilis,* 23 for *Burkholderia* sp., and 18 for *Mitsuria* sp. and 35 for control.

Nutrient depletion study

Arabidopsis seeds were surface sterilized (20% v/v bleach for 1 min followed by five washes with sterilized distilled water) and placed on germination media (100% MS and 1% sucrose) in a growth chamber under a 16 hr photoperiod. 10 cm Deepots (Ray Leach "Cone-tainer"™ SC10) were lined with Dura-Shield™ Landscape Fabric (WEED-X®, Dalen Products, Knoxville, TN) and filled to the rim with Turface (Athletics™, Profile Products, Buffalo Grove, IL). The ceramic substrate was wet, and 7 day old seedlings were transplanted into the pots and fertilize with 100% Hoagland's with Fe_2HO_4 solution (210 ppm N) (Phytotech Lab, Shawnee Mission, KS). On the day of transplanting, *B. pumilus* liquid cultures were initiated in 3 mL of Luria-Bertani (LB) medium, and grown on a 200 rpm shaker incubator at 30ºC overnight. 24 hours after culture initiation, treatment pots were inoculated with *B. pumilus* at an inoculation rate of 10^6 cells/plant. Plants were watered every day and every 3 days the treatment concentration of fertilizer was applied to each plantlet. As the requirement for optimal Arabidopsis growth was found to be 200 ppm N following every other irrigation, each of these treatments is a nutrient deficient condition for a soilless mix (Eddy, Hahn, and Aschenbeck, 2008). The total nitrogen applied over the duration of the experiment should have been 2,100 ppm N, while treatments received 1,470 ppm, 735 ppm, and 367.5 ppm N. After 3 weeks, above-ground plant biomass was harvested and placed in a drying oven for three days. A total of 43 plants were harvested for aboveground dry biomass. Once completely dry, the dry biomass data was collected. Plant biomass was weighed using a FisherScientific A-200DS scale

Statistics

The Arabidopsis RSA and above ground dry biomass data were visualized in R software (version 3.1.3 (2015-03-09) -- "Smooth Sidewalk" © 2015) to verify that there were no outliers. Levene's test was used to verify homogeneity of variance and Q-Qplot to verify normality. If assumptions of Analysis of Variance (ANOVA) were not met, the data were transformed. Least Square Means (Lsmeans) pairwise comparison of means was performed to compare each of the treatments and controls for aboveground biomass without adjustment. ANOVA assumptions could not be met using transformations for each of the RSA variables: root length (cm), root surface area (cm^2), root volume (cm^3), average root diameter (cm), or lateral root number. For this reason, a non-parametric Kruskal-Wallis test was performed to identify statistically significant differences between means. If a significant difference ($\alpha \leq 0.1$) between treatments was identified from Kruskal-Wallis output, then the difference between groups was estimated using Dunn's Test with Benjamini-Hochberg false discovery rate adjustment. Estimates of central tendency (mean, median) and estimates of variability (standard deviation) were made without adjustment.

Results

In vitro RSA modification

A total of 128 plants were analyzed using winRHIZO software (Regent Instruments Inc., Ottawa, Canada). Statistically significant differences ($\alpha \leq 0.1$) were identified for total root length among some of the treatments. For instance, *Bacillus pumilis* increased root length (μ=18.52cm, SE=1.3) compared to the un-treated control (μ=14.02cm, SE=0.72) (Figure 2, Table 3). *Mitsuria* sp. also induced a similar effect on the roots (μ=17.46cm, SE=1.09) (Table 3). Statistically significant differences were also identified for root surface area between control (μ=0.86cm^2,

SE=0.04) and *B. pumulis* (μ=1.28cm^2, SE=0.1) as well as between control and *Mitsuria* sp. (μ=1.26cm^2, SE=0.12) (Table 3, SFigure 3). For average root diameter, *B. pumulis* (μ=0.22cm, SE=0.003) and *Mitsuria* sp. (μ=0.22cm, SE=0.01) significantly increased growth compared to the un-treated control (μ=0.20cm, SE=0.003) (Table 3, SFigure 4). For root volume, significant increases were identified between control (μ=0.004cm^3, SE=0.0002) and *B. pumulis* (μ=0.007cm^3, SE=0.0007), and *Mitsuria* sp. (μ=0.007cm^3, SE=0.001) (Table 3, SFigure 5). Finally, for lateral root number, which was counted by hand for accuracy, highly significant ($\alpha \leq 0.001$) increases were identified with bacterial treatment for control (μ=11.43, SE=1.26) and *Burkholderia* sp. (μ=19.64, SE=1.45), as well as between control and *Mitsuria* sp. (μ=18.24, SE=0.9) (Figure 3, Table 3).

Nutrient depletion study

Following the completion of the in vitro RSA modification study, *B. pumilus* was selected for further investigation based on observations of longer RSA. In a growth chamber experiment, it was investigated whether *B. pumilus* modifications to Arabidopsis RSA increased nutrient acquisition ability and therefore biomass under nutrient deficient conditions. Statistical analysis without multiple testing correction identified a significant difference ($\alpha \leq 0.1$) between the biomass of the control with Hoagland's concentration 1470ppm N (mean=0.095g, SE=0.002) and the biomass for the *B. pumilus* treated group (mean=0.15g, SE=0.014) (Figure 4). No other significant differences were identified between PGPR treated and untreated plants for the Hoagland's concentrations 735 ppm or 367.5 ppm N application rates.

Table 3. Summary of statistically significant ($\alpha \leq 0.1$) changes to RSA or biomass with bacterial application in vitro and in the growth chamber.

Bacteria	In vitro	Growth Chamber
***Bacillus pumilus* CL29**	Increased total root length (cm), root surface area (cm^2), average root diameter (cm), root volume (cm^3),	Increased above ground dry weight (g)
***Mitsuira* sp. ADR17**	Increased LR number, total root length (cm), root surface area (cm^2), average root diameter (cm), and root volume (cm^3)	
***Burkholderia* sp. ADR10**	Increased LR number	

Discussion

Soil exploration for nutrient uptake is highly dependent on roots, so a major adaptation by plants to nutrient deficiencies is modifying root architecture (Li, Zeng, and Liao, 2015). Rather than waiting for plants to experience stress from nutrient deficiencies and prompt RSA changes, application of a PGPR which induces a desired RSA could increase nutrient uptake efficiency and avoid undue plant stress. In this study, we show that three bacterial species (*Bacillus pumilus* CL29, *Mitsuria* sp. ADR17 and *Burkholderia* sp. ADR10) isolated from soil which produce statistically significant changes in root system architecture. Further investigation found that one of these bacteria, *Bacillus pumilus* strain CL29, significantly promoted aboveground biomass in nutrient deficient conditions, theoretically due to increased nutrient acquisition due to increased root growth. We can suggest that the bacteria increased growth by inducing helpful RSA and not by modifying nutrients because Hoagland's fertilizer contains nutrients in plant available form.

Soil resources such as nitrogen, phosphorus, and water, are non-uniformly distributed in the soil profile, requiring that the plant root system architecture to specifically address each issue (Rogers and Benfey, 2015). RSA optimized for drought tolerance, for example, is narrower and vertically oriented (Rogers and Benfey, 2015) but root density near the soil surface is better for

environments where water is supplied by periodic rainfall events during plant development (Tron et al., 2015). Narrow and long RSA is best suited for the uptake of mobile resources such as sulfate and nitrogen which are typically found deeper in the soil profile (Kong et al., 2014). In this study, *Bacillus pumilus* CL29 significantly increased root growth without increasing LR density in vitro. In a subsequent growth chamber experiment, *B. pumilus* plantlet inoculation significantly promoted aboveground biomass under nutrient deficient conditions for the 1,470 ppm N Hoagland's application rate, but not for the more deficient, 735 or 367.5 ppm N Hoagland's application rates. These results support the use of, *B. pumilis* in situations where nutrients are scarce for increased uptake but only to an extent.

RSA optimized for immobile nutrients, on the other hand, is comprised of highly branched, but more shallow RSA. Access to phosphorus, potassium, iron, and manganese in the topsoil would be increased by lateral root branching density without increased root length (Kong et al., 2014). *Mitsuria* sp. significantly increased root growth and induced lateral root formation. *Burkholderia* sp. significantly increased lateral root formation but did not increase root growth as described by length, surface area, volume, or diameter. These results support that these PGPR could be used to induce root uptake of immobile nutrients.

There are multiple studies which have investigated the versatile genera *Bacillus*, *Burkholderia*, and *Mitsuria* which are not only found in the soil, but in some cases in the gut of humans, in dust, rocks, or in other environments (Hong et al., 2009). One example of versatility is *Bacillus* spp. being used as probiotics for both livestock and humans (Hong et al., 2009), while it is also well known for plant growth promotion by mechanisms such as inducing systemic resistance (ISR). The species used in this study, *B. pumilus,* was effective in decreasing disease severity of cucurbit wilt (Kloepper, Ryu, and Zhang, 2004; Gardener, 2004). A number of

Bacillus species have been commercially available fungicides, insecticides, and nematicides, or plant growth promotion formulations (Kaki et al., 2013). One member of the *Burkholderia* genus (*Burkholderia phytofirmans)* has been found to induce biomass in multiple plant species, including potato. Potato growth promotion was attributed to changes in biosynthesis of growth hormones indole-3-acetic acid (IAA), cytokinin (CK), and gibberellin (GA) (Kurepin et al., 2015). Some members of *Burkholderia* have also been found to control plant pathogens or act in bioremediation, but they are highly host specific, and some may pose health risks to humans (Coenye and Vandamme, 2003). *Burkholderia* sp. and *Mitsuria* sp. were found to be effective biocontrol agents against bacterial leaf spot and growth promoters. Both enhanced shoot growth and biomass in tomatoes (Cepeda and Gardener, 2012). These diverse mechanisms suggest that application of the bacteria investigated in the present study may benefit the plant by preemptively modifying RSA as well as help the plant using other unidentified mechanisms.

Although root system architecture is of interest for productivity, the mechanisms that determine RSA are poorly understood (Benitez-Alfonso et al., 2013). The model plant used in this study, *Arabidopsis thaliana* mainly forms a tap root system with a single primary root and a few post-embryonically initiated lateral roots (Kong et al., 2014) which grow at regular intervals from LR founder cells. These founder cells undergo many divisions until the tissue organization matches that of the root meristem and the LR emerges from the parental root (Lavenus et al., 2013). Various types of cell-to-cell communication are extremely important to this process (see review: (Yue and Beeckman, 2014)). Grown in vitro, Arabidopsis LR distribute evenly along the primary root controlled by locally synthesized auxin and shoot-derived auxin targeted by auxin transport, auxin signaling, and oscillations in gene expression (For a detailed review of auxin's role in lateral root development see (Lavenus et al., 2013)). Although auxin production was not

investigated in this study, it is likely a player in the mechanisms making modifications to RSA by these bacterial strains. Microbes have been known to manipulate a plant's auxin balance to stimulate growth, to colonize the plant, and increase gene expression for defense, or to increase pathogenic ability (Ludwig-Mueller, 2015). Multiple studies have also illustrated the role of bacterial volatile organic compounds (VOCs) in modification of plant RSA (Ryu et al., 2003; Gutierrez-Luna et al., 2010; Delaplace et al., 2015; Ditengou et al., 2015) though it is not believed that these bacterial strains are using VOC based on a few observational experiments with divided plates.

Conclusion

Changes in Arabidiopsis RSA and biomass attributed to PGPRs are likely unique to the bacterial strain. *Bacillus pumilus* strain CL29 significantly increased total root length, root surface area, root volume, average root diameter, and above ground dry weight (biomass) compared to un-treated control. *Mitsuira* sp. significantly increased the number of lateral roots, total root length, root surface area, average root diameter, and root volume. On the other hand, *Burkholderia* sp. significantly increased lateral root branching but did not significantly increase any other root parameter. Development of crops with improved resource uptake ability will be extremely important as pressures from crop production needs, climate change, and fertilizer resources are depleted. In agreement with a growing demand for sustainable practices, the use of PGPR could increase our ability to address these issues.

Figure 3. Plate layout for RSA experiments. Two, four day old Arabidopsis seedlings were placed at the top, and 10µL of 10^6 cells/mL bacterial suspension was plated below each plant. Plates were stored vertically with the plantlets facing up and the bottom wrapped with aluminum foil to limit root exposure to light.

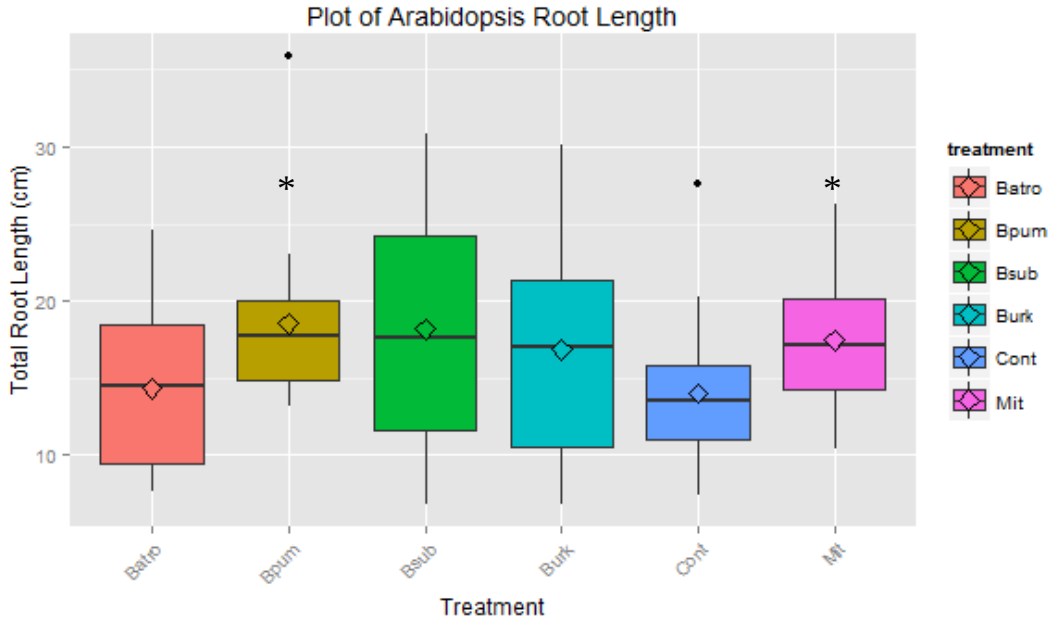

Figure 4. Arabidopsis total root length grown on petri dishes treated with and 10µL of 10^6 cells/mL bacterial suspension plated below each plant measured with winRHIZO software. A

total of 128 plantlets were analyzed including 35 control 24 *Bacillus atropheus* (Batro), 17 *B. pumilus* (Bpum), 11 *B. subtilis* (Bsub), 23 *Burkholderia* sp. (Burk), and 18 *Mitsuria* sp. (Mit). *B. pumilus* and *Mitsuria* sp. significantly ($\alpha \leq 0.1$) increased total root length.

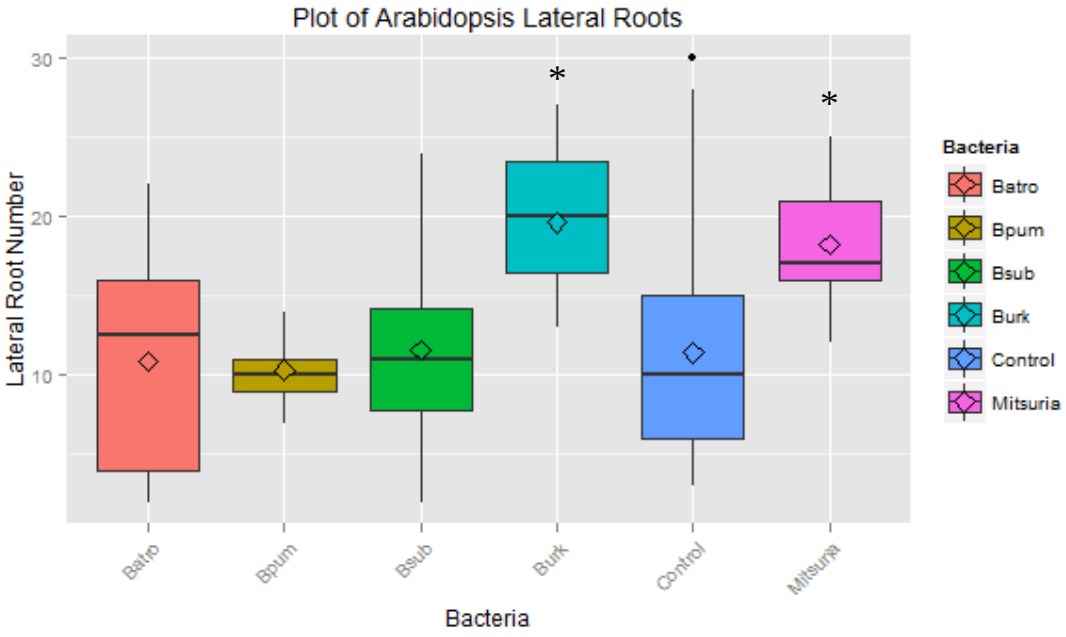

Figure 5. Arabidopsis lateral root numbers counted by hand grown in plates treated with and 10µL of 10^6 cells/mL bacterial suspension plated below each plant. A total of 118 plantlets were analyzed including 35 control 24 *Bacillus atropheus* (Batro), 19 *B. pumilus* (Bpum), 12 *B. subtilis* (Bsub), 11 *Burkholderia* sp. (Burk), and 17 *Mitsuria* sp. (Mit). *Mitsuria* sp. and *Burkholderia* sp. significantly increased LR number.

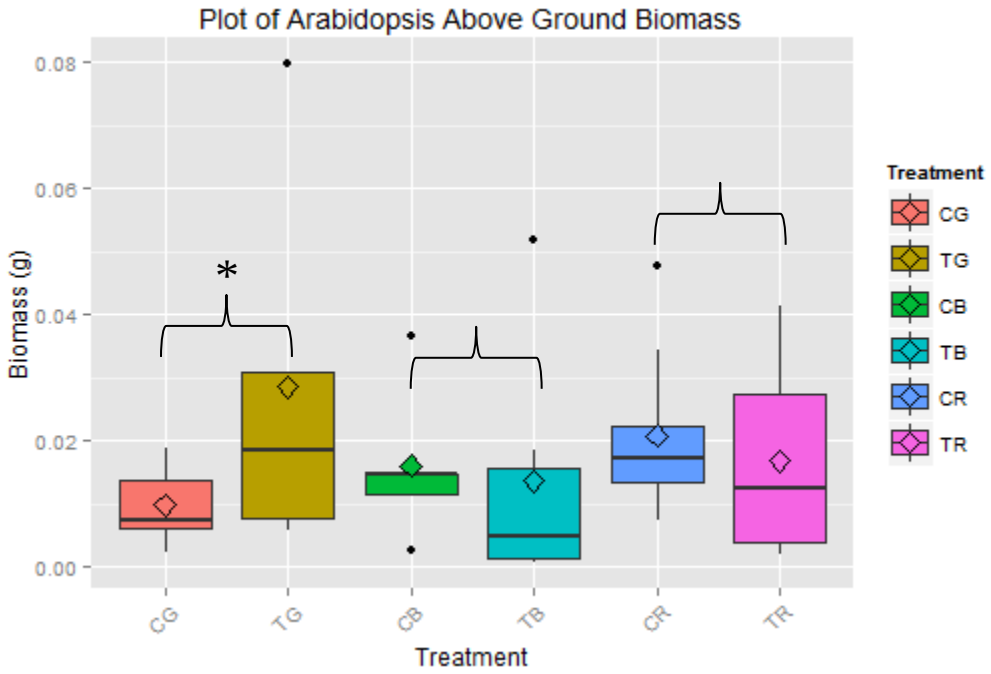

Figure 6. Arabidopsis dry weight of above ground plant parts grown under nutrient limited conditions. Controls (CB, CG, CR) were not inoculated with the PGPR, *Bacillus pumilus*, while treatments (TB, TG, TR) were inoculated. Hoagland's application rates for total nitrogen over the 3 week experiment were CG: 1,470 ppm, CB: 735 ppm, CR: 367.5 ppm, TG: 1.4700, TB: 735 ppm, TR: 367.5 ppm. Dry weight significantly increased for TG (inoculated 1,470 ppm N) compared to CG (un-inoculated 1,470 ppm N).

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CHAPTER 3 SOIL MEMORY AS A POTENTIAL MECHANISM FOR ENCOURAGING SUSTAINABLE PLANT HEALTH AND PRODUCTIVITY[1]

Synopsis

The unspecified components of plant-microbe and plant-microbiome associations in the rhizosphere are complex, but recent research is simplifying our understanding of these relationships. We propose that the strong association between hosts, symbionts, and pathogens could be simplified by the concept of soil memory, which explains how a plant could promote their fecundity and protect their offspring through tightly associated relationships with the soil. Although there are many questions surrounding the mechanisms of this phenomenon, recent research has exposed evidence of its existence. Along with evidence from observations and mechanisms related to soil memory, we report means to utilize our understanding as sustainable protection for agricultural crops and propose future research questions.

Introduction

Studies of plant fossils demonstrate a close association between plants and soil microbial symbionts since transitioning onto land, and conservation of the mechanisms modulating these

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This chapter includes the complete published manuscript for Current Opinions in Biotechnology which discussed a multigenerational soil health concept termed soil memory (Erin R Lapsansky, Arwen M Milroy, Marie J Andales, Jorge M Vivanco, Soil memory as a potential mechanism for encouraging sustainable plant health and productivity, Current Opinion in Biotechnology, Volume 38, April 2016, Pages 137-142, ISSN 0958-1669, http://dx.doi.org/10.1016/j.copbio.2016.01.014). My contributions to this publication included conceiving, developing the theme, and writing the paper, as well as final edits and submission. Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. figure 1 is now figure 7. This article is reproduced with permission, and only minimal modifications were made to meet formatting requirements. No other modifications were made. Reuse granted as per the Elsevier license terms and conditions March 21, 2016.

interactions (Taylor and Krings, 2005). The microbial metagenome of plants acts as an extra-organismal matrix enabling greater environmental adaptability, resource acquisition, internal and external defense responses, and the communication between plants necessary to ensure the fitness of a species or health of entire ecosystems (Rakovan, 2012). This indelible relationship between the plant and the soil microbiome is essential to plant health and productivity (Chaparro et al., 2012; Turner, James, and Poole, 2013; Lakshmanan, Selvaraj, and Bais, 2014). Still, the soil microbial community is diverse, and comprised of species that may be beneficial, commensal, or detrimental to plants. Plants and microbes have therefore co-evolved a tightly regulated defense system for protection that also tolerates formation of beneficial relationships (reviewed by (Anderson et al., 2010; Trda et al., 2015)).

Soil bacterial or fungal species that impart some benefit to plants are commonly known as Plant Growth Promoting Microorganisms (PGPM) – many of which have been developed for commercial distribution as soil amendments for implementation in agricultural systems (Hjort et al., 2014). PGPM that are introduced into soils must be able to colonize the area around (rhizosphere) or directly inside (endophytes) the roots, compete with other microbes for limited resources, and persist in the soil environment (El-Mougy et al., 2012). Enhancing soils with the application of beneficial microbes therefore produces inherently variable results, as bacteria can be unpredictable in terms of establishment and degree of plant growth promotion (Mazzola, 2007). Sarma *et al*. (2015) compiled a thorough list of the microbial consortia studied for their anti-phytopathogenic activities (Sarma et al., 2015). Preferentially, the development of suppressive soils, or soils where pathogens are present yet their impact on the host is significantly decreased (Penton et al., 2014), offers an alternative to improving crop health and yield.

In this review, we discuss current advances in knowledge of microbial plant growth promotion and defense, suppressive soils, and microbial applications in agroecosystems. Using recent findings on this topic, we propose that soil microbial communities convey attributes of specificity, heterogeneity, and growth promotion in soil that could be inherited by future crop cycles, much like mammalian microbial colonizers are passed to their offspring (Koenig et al., 2011). The maturation and maintenance of the rhizosphere community is similar to the development of the human microbiota: colonization begins at birth, and as the infant matures, the microbial community increases in population size and complexity (Kaiko and Stappenbeck, 2014) – seeded by intimate contacts with caregivers and the environment (Schloss et al., 2014). Microbes acquired by individuals to successfully adapt to their environment are then passed down to offspring (Koenig et al., 2011). We document that the current literature displays evidence that a similar phenomenon may occur in plants through a kind of 'soil memory', and review practices which we believe can encourage the phenomenon, plus identify intriguing areas for future research.

Choice Mechanisms of PGPM Activity

In addition to nutrient supplementation by microbes, regulation of plant hormones either by microbial synthesis or degradation, is a simple yet effective way in which symbionts can decrease abiotic stress symptoms caused by drought, salinity, or heat stress to maintain health of host plants (Liu et al., 2013; Khan, Waqas, and Lee, 2015). Reduction of abiotic stress effects by microbes may also occur via influencing plant genetics as opposed to direct molecular interventions; a myriad of genes related to stress tolerance, metabolism, and pathogenesis were differentially expressed when drought stressed plants were inoculated with PGPM (Choi, Kang, and Kim, 2013; Lim and Kim, 2013). Interestingly, there is new evidence that this type of

genetic manipulation in plants is caused by microbial communication proteins released into the soil and not solely by microbial presence (Salas-Marina et al., 2015). Mycorrhizal fungi can also indirectly help plants deal with biotic stress by functioning as conduits for transfer of molecular messages between a stressed plant and a neighboring plant not yet under siege, which can then preemptively establish defensive strategies (Babikova et al., 2013; Song et al., 2014). In addition to these indirect mechanisms of plant defense, PGPM impart direct defense against herbivores through toxic alkaloid production (Panaccione, Beaulieu, and Cook, 2014), and against opportunistic phytopathogens by way of antibacterial (Huang et al., 2014), antifungal (Tanaka, Ishihara, and Nakajima, 2014), and degradative enzyme (Blaya et al., 2015) secretions. If any of these, or similar microbial chemical secretions are long lasting in the soil, their effects could be imparted to future plant generations.

Soil Memory as a Potential Mechanism

Certain plants have the ability to pass on endophytic PGPM acquired from the soil to their offspring; strawberries can pass microbes through their stolons (Guerrero-Molina, Winik, and Pedraza, 2012), while several forb species pass them directly through their seeds (Hodgson et al., 2014). It is likely, therefore, that plants have also developed similar mechanisms to pass on free-living PGPM, or a specific dynamic soil microbial community to their offspring as a means of imparting the adaptive advantages developed during their life to their successors. Recently, Panke-Buisse *et al.* (2015) selected for a microbial community which altered plant flowering time in multiple hosts demonstrating that a stable microbial community can be developed to modify desired plant traits (Panke-Buisse et al., 2015). Free-living soil microbes are likely prominent players in soil memory; because they exist in the soil even when a host plant is absent, they would be a long-lasting utility available to a plant at the moment of establishment (Salas-

Marina et al., 2015) (Figure 1). However, especially in the case of microbial species unable to live without a host, the organic chemicals microbes release to manipulate plants could be incorporated into stable soil organic matter (Cotrufo et al., 2015) able to impart influence on future plant generations as they get scavenged from the soil. Likewise, the same could be said for the rhizodeposits released from plant roots (Badri et al., 2013) that can rapidly alter the soil microbial community dynamics (Yuan et al., 2015) to support their various needs during different developmental stages (Chaparro, Badri, and Vivanco, 2014). These chemical signals released into the soil may influence the establishment of future seedlings (Uddin et al., 2014), their ability to form relationships with certain PGPM, or possibly expression of advantageous adaptive phenotypes (Figure 7).

Through the recent work of Ghalambor *et al.* (2015) we can postulate that if the traits expressly brought about by the relationship between the soil microbial community and their host plants increased the capacity of both to adapt, then those changes brought about in the plant and the associated microbial community could lead to reciprocal evolutionary changes (Ghalambor et al., 2015). It is often documented that micro-evolution in the plant-microbe interaction occurs in low-nutrient environments, for example (Hartmann et al., 2009). Shi *et al.* (2015) noticed a consistent microbe assemblage pattern in the rhizosphere of *Avena fatua* that suggests that the two have co-adapted over many generations of interactions (Shi et al., 2015). Additionally, the plant's manipulation of the soil microbial community dynamics and functions may be profound enough that the changes could be present after the plant's influence is gone, affecting the next generation of plants and their ability to adapt and thrive. An example of this was identified in pine seedlings grown in soil inoculum from beetle killed pine stands. Seedlings were found to produce less of the monoterpenoids needed to fight off biotic attackers than those grown in soil

inoculum from undisturbed pine stands – a trait correlated with a reduced abundance of a certain ectomycorrhizal fungi in the beetle kill stand inoculum (Karst et al., 2015). In this specific instance, it would appear that whatever changes the attacked trees brought about in the soil left seedlings more vulnerable to future attacks. It is worthy to note, however, that the pine seedlings grown with fungal inoculum from the disturbed stand still had significantly higher monoterpenoid concentrations than those grown with no inoculum (Karst et al., 2015). These results are therefore evidence to support the theory that a plant can have altered expression of defensive strategies when exposed to the soil microbial community assemblage formed by predecessor plants while they were under biotic attack, even when those predecessors are no longer present.

Understanding Suppressive Soils

An area of interest to sustainable agriculture research is the phenomena of suppressive soils. Suppressive soils are those that decrease or prevent disease occurrence despite the presence of a pathogen, a compatible plant host, and favorable environmental conditions (Penton et al., 2014). We postulate that suppressive soils are formed though the soil memory mechanism, therefore the current understanding of the process is reviewed here. Suppresiveness is categorized as either general or specific. General suppressiveness is directly correlated to a robust microbial activity, which prevents the successful establishment of a pathogen with its plant host. Soils high in organic matter usually harbor greater organism density and therefore exhibit general suppressiveness (Cook, 2014). In contrast, specific suppressiveness involves a microorganism or a group of microorganisms that act by antagonizing the pathogen through antibiosis. Many investigations have come to the conclusion that monoculture farming induces specific disease suppression (Almario et al., 2014; Wei et al., 2015). Specific disease suppression

can also be highly dependent on crop identity and management practice, such is the case for insect pathogen and biocontrol fungal genus *Metarhizium* (Kepler et al., 2015). After establishment, certain soils can maintain disease suppression under well-defined crop rotation systems. The ability to maintain suppression despite crop rotation is evidence of either durable microbial communities or persistent chemical signals in the soil acting on future generations. Long-lasting specific suppression of *Fusarium oxysporum*-mediated wilt in flax and other susceptible crops by soil microbes (Janvier et al., 2007), and intercropping cultivation of corn and black-eyed pea against *Fusarium solani* CFF109 (Barros et al., 2014) are also examples of soil memory. In both cases, suppression was accredited to a more diverse microbiome attracted and supported by diversified host availability.

In addition, research has shown that different soil communities can be suppressive against the same pathogen (Adam et al., 2014; Hjort et al., 2014; Shen et al., 2015). It is generally accepted that the mechanism of suppression in these soils is due to microbial production of enzymes with antiphytopathogenic activity (Hjort et al., 2014). Recently, however, Chapelle *et al.* (2016) investigated soils suppressive to *Rhizoctonia solani* using metatranscriptomics, and proposed that the pathogen was activating survival strategies in members of the rhizosphere which consequentially reduced *R. saloni* growth, induced plant resistance, and activated other microbes able to fight the fungal pathogen (Chapelle et al., 2016). The resulting stress changed the composition of the rhizosphere and its functions promoting the activation of antagonistic traits in soil microbes as well as plant resistance responses (Chapelle et al., 2016). That these suppressive mechanisms are accomplished by a wide range of microbial consortia suggests that, with correct management, any soils could become suppressive and productive through supporting soil memory.

Promotion of Soil Suppression

The development and maintenance of healthy, pathogen-suppressive soils can be a goal for productive and sustainable agriculture. Many problems in agriculture related to soil pests and diseases can be linked to poor management practices. Farming practices which do not protect soil health lead to poor drainage, structure, organic matter, and fertility, and have negative impacts on the soil microbial community (Dorr de Quadros et al., 2012; Ito, Araki, and Komatsuzaki, 2015). Tilling often requires complete vegetative removal between crops and leads to soil erosion and degradation as well as drastic changes in soil microbial community composition (Dorr de Quadros et al., 2012; Kepler et al., 2015). Because evenness of the soil community is a component of soil suppressiveness, poor soil management practices and the application of pesticides, fungicides, and fertilizers, disrupts microbial community balance (Qiu et al., 2014) as well as inhibits the mechanism of soil memory by encumbering sufficient interaction between the plant and soil microbial community. Poor soil quality also inhibits the ability of plants to select a specific microbial community (Tkacz et al., 2015). Utilizing the mechanism of soil memory to develop suppressive soils requires cropping systems which support soil microbial diversity and allow prolonged interaction time between crops and soil communities.

There is no recipe for creating a suppressive soil because there are so many interrelated components. For example, monocropping of soybean decreased the incidence of disease symptoms and pest populations compared to rotation cropping systems (Wei et al., 2015). Continuous cropping of banana for over 14 years resulted in less than 14% disease incidence despite proximity to orchards with diseased soil (Shen et al., 2015). Discordantly, both monocropping and intercropping cultivation systems developed suppressive ability against root

rot in cassava, attributed to increasing soil community diversity and nourishing antagonistic microbes with organic matter amendments, respectively (Barros et al., 2014). No-till practices have increased soil suppression, soil carbon content, and soil community diversity (Dorr de Quadros et al., 2012; Ito, Araki, and Komatsuzaki, 2015). Crop residue decomposition in no-tillage systems support soil memory by progressively releasing nutrients to support the inherited soil community for future generations (Dorr de Quadros et al., 2012). To target specific antagonistic microbes, the addition of pure chitin or feather or hoof meal (waste products containing chitin), were demonstrated as effective at promoting suppressive soils, specifically by increasing abundance of *Lysobacter* spp., a relatively uncommon genus, antagonistic to *Rhizoctonia solani* (Postma and Schilder, 2015). However, evidence suggests that multiple members of co-adapted microbial taxa are responsible for crop health and protection (Mendes et al., 2011; Postma and Schilder, 2015), and supporting a community of beneficial microbes would therefore better suit the needs of infected fields.

Concluding Remarks

Despite continuing research on the topic of suppressive soil, there are many gaps in our understanding of the phenomenon. Our understanding of plants as chemical factories responsible for coordinating many underground interactions (Badri et al., 2009) prompts exploration of spatial relationships, soil environmental variables, and soil chemistry requirements of suppressive soils (Barros et al., 2014). Additionally, while many studies have explored the dynamics of suppressive soil microbial communities, few functional evaluations have been conducted (Almario et al., 2014). Mendes *et al.* (2011) proposed that pathogen pressure is required for suppressive soil development (Mendes et al., 2011), however, Wei *et al.* (2015) was able to develop suppressive soil without pathogen pressure (Wei et al., 2015), suggesting that

pathogen presence may speed up development, but is not required for suppressive soil formation. The most crucial questions revolving around the concept of soil memory include how long suppressive soils can be maintained in a field, and if there is more evidence of plants passing down specific microbial communities to their offspring. Like humoral immunity, soil memory seems to display features of specificity, heterogeneity, and the ability to transfer defense mechanisms to offspring. Whether soil microbes are passed down on the seed coat, through recalcitrant root exudates, or durable community structure and microbial dormancy warrants further investigation. Comparable to the laborious process of biofertilizer or biopesticide development (Vassilev et al., 2015), the development of suppressive soils may be a more cost effective and efficient alternative to microbe application. Furthermore, soil memory may reduce or eliminate the need for reapplication, producing durable results following an initial investment.

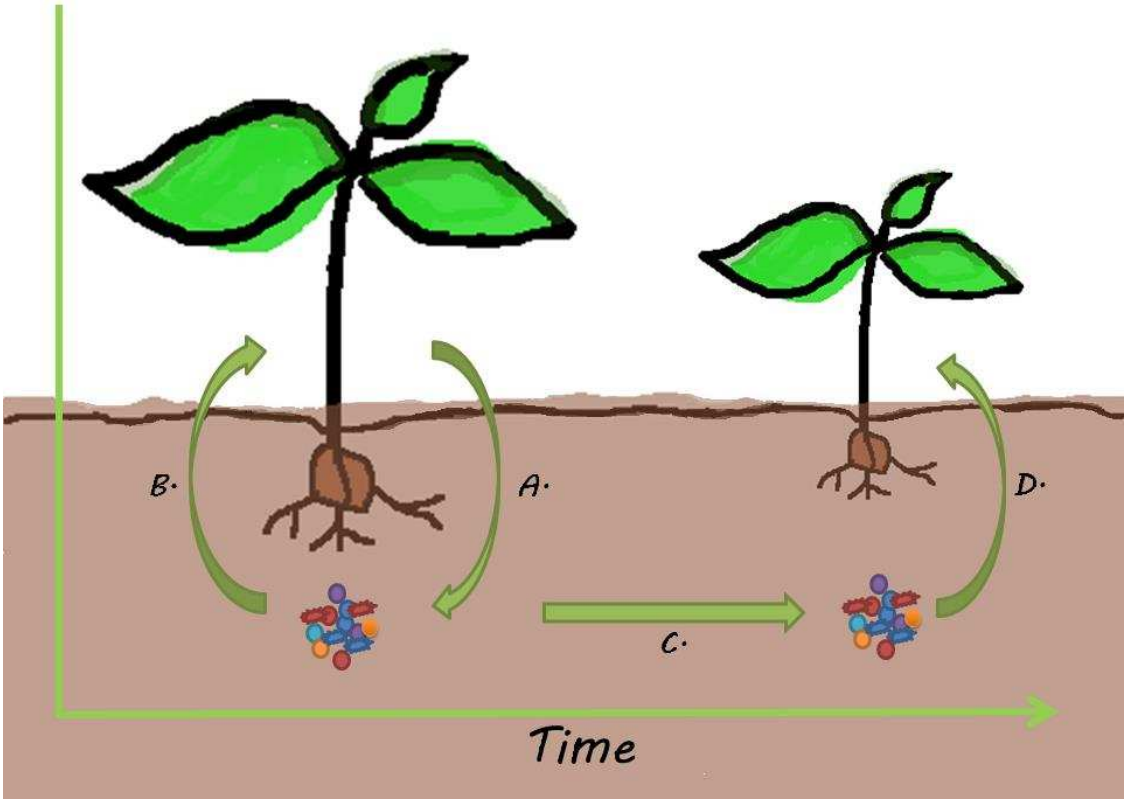

Figure 7. Schematic representation of soil memory concept where predecessor plants release molecular signals into soil to manipulate soil microbial community dynamics (A). The resulting shift then results in the release of molecular signals and resources from the microbes, which elicit phenotypic changes in the plant, and affect its ability to adapt to environmental stressors (B). Over time, even without plant presence, exudates from both the plant and microbial community could persist in soil, along with remnants of the microbial assemblage carefully culled through the exchange (C). These factors left in the soil can then impart influence on the phenotypic expression, and therefore adaptability of new plant lines naïve to the environment and the adaptive exchanges between the microbes and predecessor plants (D).

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CONCLUSION

Increasing interest in the study of the rhizosphere has resulted in the discovery of many compounds and microbes with potential to aid in feeding the growing global population. The studies conducted for this thesis identified four possibly useful bioactive root exudate compounds as well as three possibly useful plant growth promoting rhizobacteria (PGPR). These results suggest that they may be used in agriculture to increase plant productivity in certain environments. Additionally, these compounds and microbes could play roles in soil memory to pass benefits on to future plant generations.

The analysis of root exudate relative abundances revealed twenty three compounds that were changing in exudation level over the growth of Arabidopsis. Of these significantly changing compounds, GABA, levoglucosan, maleic acid, galactanol, N-acetylaspartic acid, glutamic acid, adipic acid, malic acid, and fumaric acid were subjected to further investigation to test the hypothesis that exudates changing in expression level were bioactive compounds. The results confirm this hypothesis; four exudates (fumaric acid, adipic acid, levoglucosan, and N-acetylaspartic acid) were found to induce significant changes in the growth of Arabidopsis, and two (adipic acid, levoglucosan) induced changes in maize. It is possible that the other compounds are also bioactive, but that their activity is directed towards the rhizosphere microbes or other organisms.

The investigation of PGPR on plant growth identified modifications to root system architecture (RSA) induced by plant growth promoting rhizobacteria (PGPR). Five microbes (*Bacillus subtilis* strain T2, *Bacillus atrophaeus* strain LSSC3, and *Bacillus pumilus* CL29, *Burkholderia* sp. ADR 10 and *Mitsuaria* sp. ADR17) were studied for their effects on the roots

of Arabidopsis growth in vitro. Three microbes, *Bacillus pumilus* strain CL29, *Mitsuira* sp., and *Burkholderia* sp., significantly altered the root structure. To test the hypothesis that these root architecture changes could aid the plant in nutrient deficient conditions, *Bacillus pumilus* CL29 was used in a growth chamber experiment. Because 200 ppm N following every other irrigation is the requirement for optimal Arabidopsis growth (Eddy, Hahn, and Aschenbeck, 2008), the total nitrogen recommended for our experiment would be 2,100 ppm N. Instead, nutrient deficient treatments received 1,470 ppm, 735 ppm, and 367.5 ppm N. Under somewhat limiting nutrient conditions (1,470 ppm N), *B. pumilus* inoculation increased Arabidopsis biomass compared to the un-inoculated control, supporting the hypothesis that the increased root growth would support nutrient acquisition. The plantlets were only inoculated once, and colonization of the roots was not confirmed, but the growth promotion suggests that the bacteria persisted in the soil-less substrate for the entirety of the experiment. This colonization should be confirmed before the strain is investigated further for agricultural application purposes. It would also be important to test the strain on multiple economically important plants as this study only investigated the impact on Arabidopsis.

The findings of these studies could be used to support the hypothesis of soil memory. The root exudates being excreted at different levels over the development of Arabidopsis may be designed to recruit beneficial microbes to the rhizosphere to support the growth of offspring. They may also persist in the soil long enough to directly support Arabidopsis fecundity. The impact on the rhizosphere and the persistence of these compounds in the soil absent plants should be investigated further. The identification of PGPR which significantly modified root system architecture also lends itself to soil memory. Free living and spore forming bacteria are able to persist in bulk soil, and would be able to immediately colonize and impart benefit to

germinating seedlings, possibly the offspring of the plant which support their survival. Further investigation into the persistence ability of these bacteria is needed to support this hypothesis.

S Table 1. Root exudate compounds collected over a period of 3 days during distinct developmental stages (7-10 days, 14-17 days, 21-24 days and 28-31 days). Compounds were detected using GC-MS. Numbers indicate the average area under the curve of three replicates. Unidentified compounds not shown.

Compound	7-10 days	14-17 days	21-24 days	28-31 days
(s)-(+)-mandelic acid	83.33333333	104	104.3333333	870
xylose	548.6666667	409	952.3333333	7772
xylonolactone NIST	511.6666667	185.3333333	3853.666667	15728.33333
xylonic acid	98.66666667	73.66666667	294.6666667	7177.333333
xanthine	108.6666667	69	219.6666667	2000
valine	42631.66667	5510.333333	132138	266743.3333
urocanic acid	98.66666667	83.33333333	323.3333333	1024.333333
uridine	138.3333333	83	1319.666667	3523.333333
uric acid	112.6666667	87.66666667	565.6666667	389
urea	45373.33333	6069	445085.3333	573560.6667
uracil	969	505.3333333	2514.666667	59205.33333
tyrosine	6150	635	23048.33333	107166
tryptophan	1233.333333	184.6666667	16494.66667	183897
thymine	95.33333333	76.33333333	203.6666667	12209.33333
threonine	11678.33333	1768.333333	42357.66667	107163
threonic acid	1934.666667	505.3333333	15608.66667	84286.66667
threitol	1286	147.6666667	3660.666667	10544.33333
tagatose	142	62.66666667	219	1193.333333
sucrose	3557.333333	44098.33333	400.3333333	752.6666667
succinic acid	2629.666667	907.3333333	13643	62904.33333
succinate semialdehyde	205.6666667	212	628	2232.333333
suberyl glycine	1876	131.6666667	196686.6667	508497.3333

stearic acid	74640.33333	52620.66667	73804.33333	83236.66667
sophorose	82.33333333	90	305.3333333	1788.333333
shikimic acid	716.3333333	178.3333333	8939	22176.33333
serine	47536.33333	3831.333333	172034.6667	223634.6667
sarcosine	1284.666667	1309.333333	819.3333333	4066
salicylaldehyde	157.3333333	123	125.3333333	301.6666667
saccharic acid	88	83.66666667	470.3333333	4960.333333
ribitol	745.6666667	78.33333333	1520	2039.333333
raffinose	129.3333333	113	678.3333333	1147
quinic acid	69.33333333	70.66666667	85	367.3333333
pyrazine 2,5-dihydroxy NIST	128.6666667	98.33333333	360.3333333	1309.333333
putrescine	279.3333333	251	8059.333333	278834.6667
pseudo uridine	572.3333333	237.3333333	2823	21670.33333
proline	7468	1319.333333	30929.66667	119552.6667
pipecolic acid	317.3333333	381.3333333	867	5196.666667
phosphoethanolamine	402.6666667	102	2939.666667	10987.66667
phenylalanine	4627.333333	774.3333333	30818	385334
phenylacetic acid	88.66666667	102	75	227
pelargonic acid	1092	1294.666667	1586.666667	1951
parabanic acid NIST	732.6666667	346	5548	19612
palmitic acid	11222	8704.666667	11899.33333	13096.66667
oxoproline	306424	22495.33333	1732061.667	1841333
oxalic acid	2671.333333	1687.333333	3438	11235
orotic acid	102	83	207.6666667	1180
ornithine	3858	460	100245	473911
oleic acid	78.66666667	62	93.66666667	106.3333333
O-acetylserine	174.6666667	141.6666667	259.3333333	428.6666667
nicotianamine	191.6666667	104.6666667	762	28591
N-methylalanine	752.3333333	286.3333333	1938.333333	7753
n-epsilon-trimethyllysine	91.33333333	81.33333333	88.66666667	555
N-acetylputrescine	230.3333333	70.66666667	4172	24747.33333

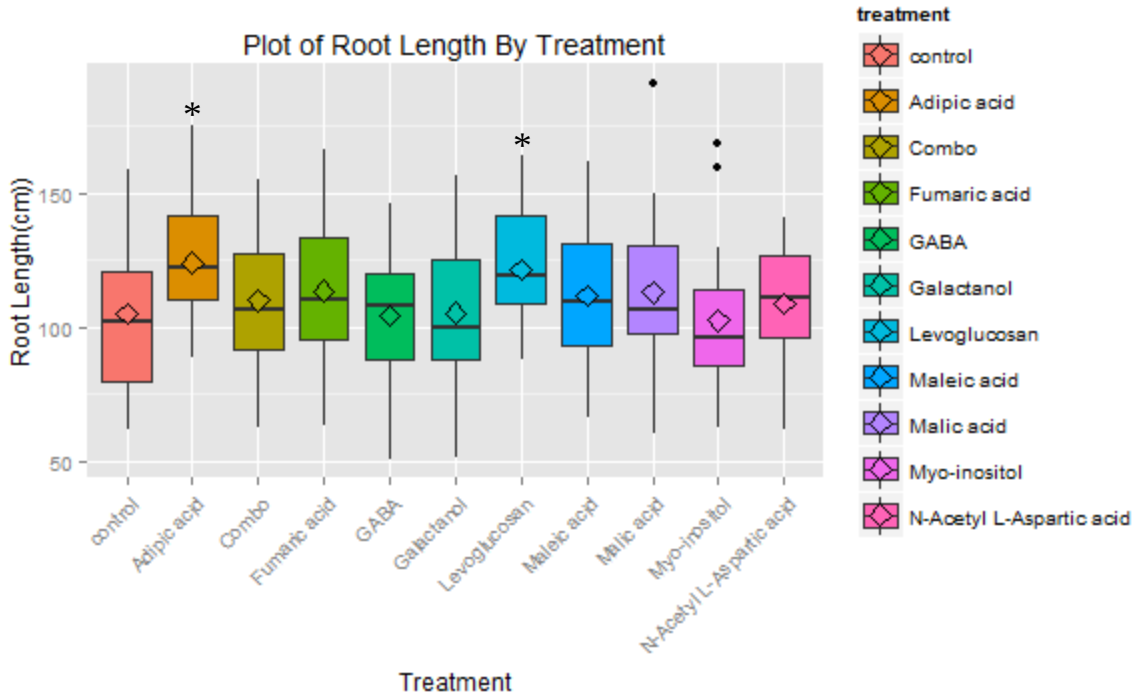
N-acetylornithine	1115.666667	214.3333333	12310.33333	11704
N-acetylmannosamine	1734.333333	123	9850	30913
N-acetyllysine	807.6666667	665.6666667	4434	17674.66667
N-acetylglutamate	156	132.6666667	4105	3964.333333
N-acetylgalactosamine	129.3333333	97.66666667	273.3333333	1373
N-acetylaspartic acid	94.66666667	101.6666667	285.6666667	587
N-acetyl-D-mannosamine	409	83.66666667	1613	3084.333333
N-acetyl-D-hexosamine	1245	296	8459	25593.66667
methylhexose NIST	139	120.3333333	14200.33333	9743.666667
methionine sulfoxide	593.3333333	266.3333333	5726.666667	31289
methionine	739.6666667	158.6666667	8392	60811
methanolphosphate	402.3333333	117.3333333	83642.33333	8046.666667
melibiose	240	226	1448.333333	2544.333333
maltotriose	110.3333333	105	176.6666667	1375
maltose	574.6666667	191	526.6666667	1285
malonic acid	6271.666667	3270	81216.66667	930856
malic acid	24372.66667	539	149607.3333	340263.3333
maleic acid	1665.333333	749.3333333	11363.66667	21257
lysine	1252.666667	516.6666667	14762.66667	138987.6667
levoglucosan	166	169.6666667	461	789
levanbiose	253.3333333	79	1136.666667	1612.333333
leucine	17693.33333	2705	56211	143525.3333
lauric acid	774.3333333	446.6666667	1268.666667	5757.333333
lactobionic acid	500.3333333	109.6666667	2825	14986
kynurenine	115.3333333	76.66666667	336	2442.333333
isothreonic acid	135.3333333	159.3333333	950.6666667	6660
isonicotinic acid	634	178.6666667	2952.666667	17211.66667
isoleucine	31190	3025.333333	97824.33333	181322.3333
isocitric acid	106.3333333	70.33333333	1060.666667	14015.33333
inulobiose	283.3333333	110.6666667	16647.33333	28349.66667
inositol-4-monophosphate	129	83	201.3333333	353.3333333

inositol myo-	5668.333333	401.6666667	36903	108351
indole-3-acetate	116	76	221.6666667	739
idonic acid NIST	88	83.66666667	439.3333333	4916.666667
hydroxylamine	6273.666667	4245.666667	6028.333333	6881
homoserine	131.3333333	88	283	4502
homocystine	187	93.66666667	1368.666667	21850.66667
histidine	2760.333333	574	50265.66667	321781.3333
hexuronic acid	94	115.6666667	182	1879.333333
hexose 2-deoxy	398.3333333	163.3333333	2198.666667	16645.66667
guanosine	69	86	679.6666667	1058
guanine	247	71.33333333	941	7074.333333
glycolic acid	449	1805	1961	6416
glycine	54039.66667	7726.333333	130094.3333	373571.6667
glycerol-beta-phosphate	288	100.6666667	648	1731
glycerol-alpha-phosphate	274.6666667	82.33333333	689.6666667	24845
glycerol-3-galactoside	8189	766.3333333	327435.6667	443170
glycerol	37780.66667	9196	42759	125250.6667
glyceric acid	5907	1183.666667	18668.66667	160259.3333
glutaric acid	189.6666667	169.3333333	553.6666667	3344.666667
glutamine	91583.33333	8038.333333	495571.3333	1006709.667
glutamic acid	27837.33333	1055	443097.3333	908796
glucose-6-phosphate	121.3333333	89.66666667	185	907.6666667
glucose-1-phosphate	378.3333333	203.3333333	841.6666667	8155.666667
glucose	33190.66667	42754	39148.66667	95307.66667
gluconic acid	128.3333333	82.33333333	341.6666667	15779.33333
glucoheptulose	232.3333333	88.66666667	1372.333333	6343.666667
galactonic acid	323	97.33333333	1442.333333	16291.66667
galactinol	7952.666667	1217.666667	129744.6667	239234.3333
GABA	44071	2723	684531.6667	954341
fumaric acid	6787	1568.333333	124750.6667	284439.3333
fucose + rhamnose	11920.66667	431	33581	90239.33333

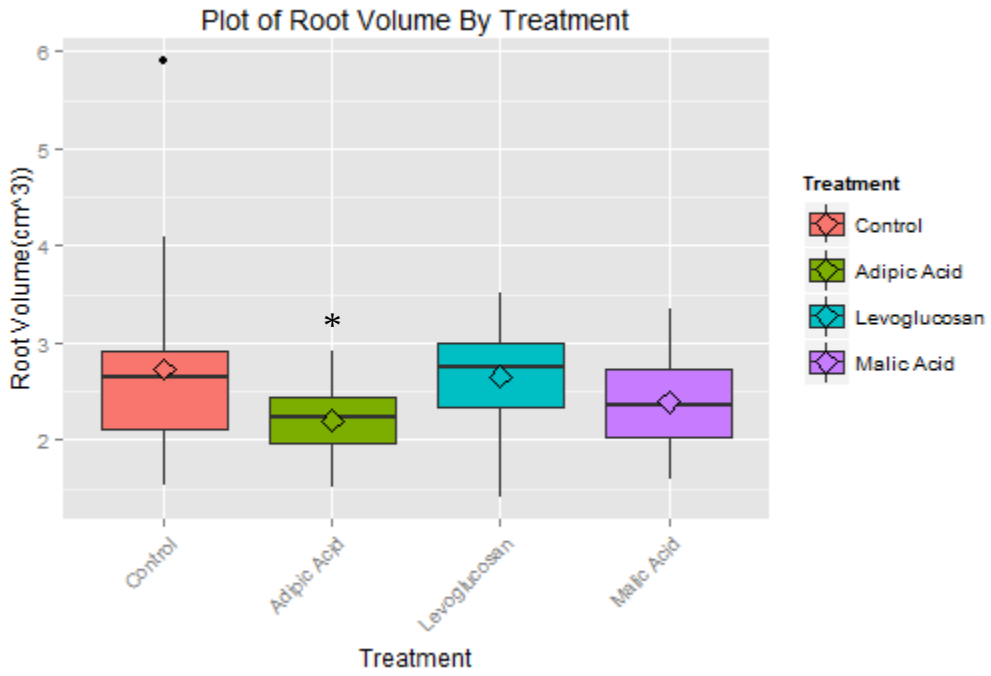
fucose	3438.333333	204.6666667	12153	35312
fructose	143989	23591	80802	107137.6667
ethanolamine	5002.666667	1228.666667	66473.66667	1205141.333
erythrose	236.6666667	171	233	469.6666667
erythronic acid lactone	5552.333333	1139.666667	22975	14368.66667
erythritol	1084	127	6751	27137.33333
epsilon-caprolactam	115	103.3333333	204.3333333	586
enolpyruvate NIST	112	95	124.3333333	415.3333333
dihydroxyacetone	95.66666667	64.33333333	334	366.3333333
cystine	294.3333333	90.66666667	939.3333333	11258.33333
cysteine-glycine	120.6666667	77.66666667	912.3333333	3688.666667
cyclohexylamine NIST	11608.33333	2502	5867.666667	12764.66667
cyano-L-alanine	790.6666667	201	9444.333333	251414.3333
conduritol beta expoxide	86	66.33333333	81	1080.333333
citrulline	488	103.6666667	9206.333333	26738.66667
citric acid	1624.333333	267.6666667	95268.33333	661764.6667
citramalic acid	125.3333333	99.66666667	322	3953.333333
cellobiotol	242	91.33333333	618.3333333	5727.333333
cellobiose	386.3333333	153.6666667	865.6666667	2950.333333
butyrolactam NIST	852.6666667	336	7312.666667	18264.33333
biuret	315.6666667	134.6666667	1421	7975.333333
beta-gentiobiose	137	101.6666667	588.3333333	1759.333333
beta-alanine	1614	124.3333333	5721.333333	18303
benzoic acid	779.6666667	857.6666667	1224	9455.333333
azelaic acid	144.3333333	85	277	1456.333333
aspartic acid	22713	1263.666667	258860	597693.3333
asparagine	83804.33333	5210	825318.3333	1221169.333
arabitol	125.3333333	86	442.3333333	1551.666667
arabinose	1806	433	3006.333333	8041.333333
alpha ketoglutaric acid	1388	103.3333333	11911.66667	86821.66667
allantoic acid	16646	2786.666667	146925.3333	378878

alanine	53230	5209	156203	437815.3333
agmatine	79	118	256	2743.333333
adipic acid	422.6666667	484	765.6666667	1022
adenine	498.3333333	134.6666667	2833	8795.333333
aconitic acid	92	69	843.6666667	8445
5'-deoxy-5'-methylthioadenosine	98.66666667	82.66666667	104	1115
5-hydroxynorvaline NIST	291.6666667	121.3333333	2710	19864.66667
5-aminovaleric acid	93.33333333	86	363.6666667	6419.666667
4-hydroxybutyric acid	119.3333333	100.3333333	324.3333333	2342
4-hydroxybenzoate	317.3333333	255	914.6666667	17216.33333
4-aminobenzoic acid	66.33333333	100.3333333	182.6666667	622
4-acetamidobutyric acid	84.66666667	85.66666667	107	3344.666667
3-phenyllactic acid	111	99.33333333	100.3333333	6670
3-methoxytyrosine NIST	101.6666667	87.33333333	311.6666667	1945.333333
3-hydroxypropionic acid	176	782.3333333	4141	16708.66667
3-hydroxybutanoic acid	213.6666667	202.6666667	515.6666667	972.3333333
3-hydroxy-3-methylglutaric acid	706.6666667	162	872.3333333	22393
3-aminoisobutyric acid	296	103.3333333	1057	7186.333333
2,6-diaminopimelic acid	85.33333333	63	106.6666667	397.6666667
2-monopalmitin	98.33333333	70	210.6666667	925
2-methylglyceric acid NIST	136.6666667	173.6666667	258.3333333	1251.666667
2-ketoisocaproic acid	267.6666667	246	292.3333333	733.3333333
2-ketobutyric acid	196	209.6666667	1199.666667	68592
2-isopropylmalic acid	89.66666667	96.33333333	128.6666667	3557.666667
2-hydroxyvaleric acid	232.3333333	538.3333333	274.3333333	7904.333333
2-hydroxyglutaric acid	1008	429.6666667	13927	88645
2-hydroxybutanoic acid	430.6666667	220	235.6666667	1629
2-deoxytetronic acid	425	428	463.6666667	1824.333333
deoxythreitol NIST	678.6666667	313.6666667	1975	18029
2-deoxyerythritol	195	88.33333333	3528	9392
2-aminoadipic acid	316	97.66666667	11909.33333	123208

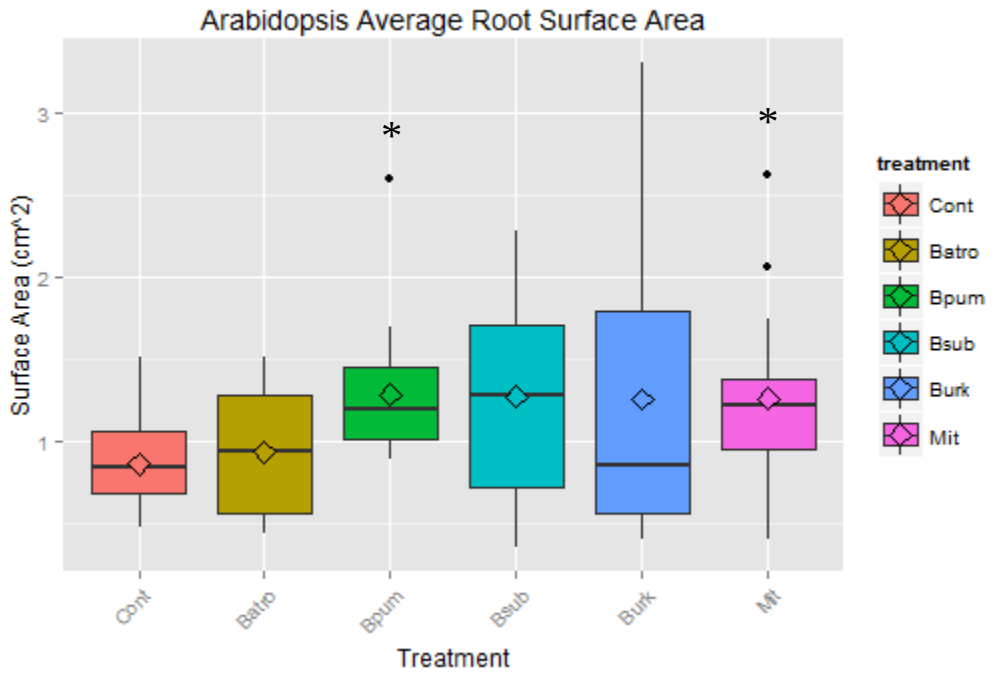
1-kestose	126.3333333	120	138.6666667	934
1-deoxyerythritol	609.6666667	104	6337	16986.66667
199177 carbohydrate	225.6666667	98.33333333	2401.333333	12774.33333



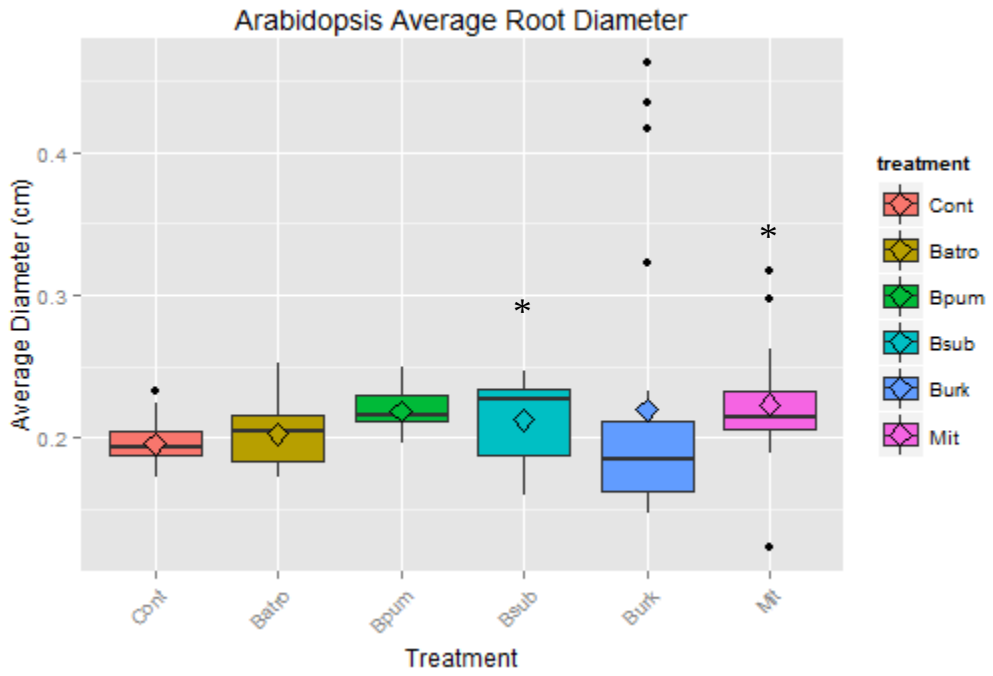
SFigure 1. Total root length by treatment for Arabidopsis in vitro. From 19-27 plants were analyzed for each treatment and the control for a total of 266 plants assessed. Adipic acid, and levoglucosan increased root length significantly ($\alpha \leq 0.1$).



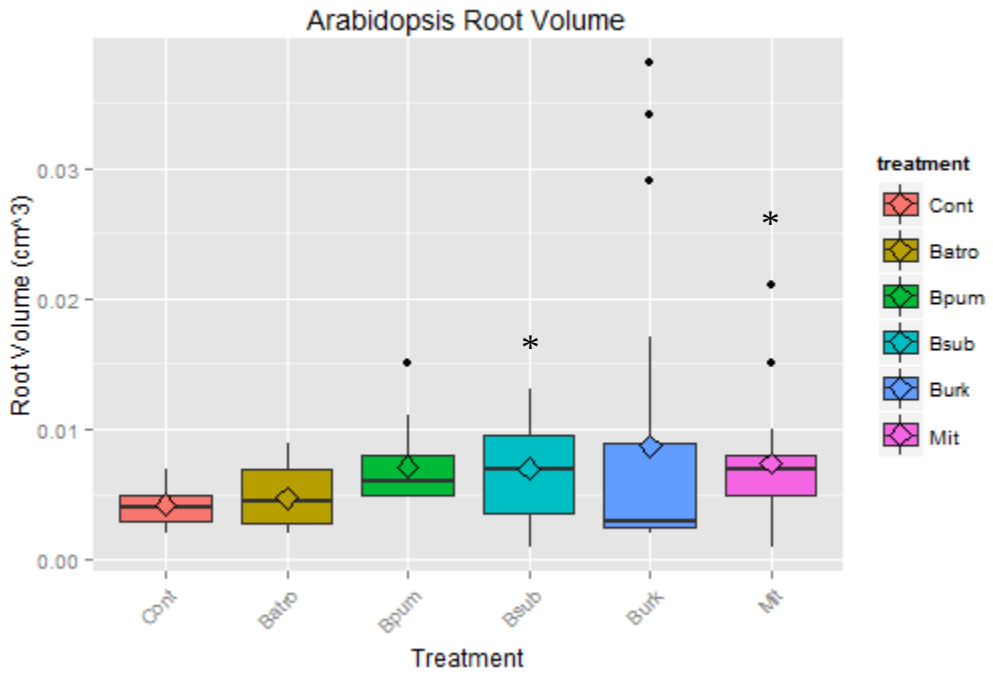
SFigure 2. Average root volume (cm3) by treatment for maize grown in the greenhouse. Twenty plants were evaluated for each treatment and the control totaling eighty maize plantlets analyzed. Adipic acid significantly decreased average root volume compared to control ($\alpha \leq 0.1$).



SFigure 3. Arabidopsis average root surface area in response to bacterial inoculation. 128 plantlets were analyzed using root architecture imaging software; 35 control, 24 for *Bacillus atropheus* (Batro), 17 for *B. pumilus* (Bpum), 11 for *B. subtilis* (Bsub), 23 for *Burkholderia* sp. (Burk), and 18 for *Mitsuria* sp. (Mit). *B. pumilus* and *Mitsuria* sp. significantly ($\alpha \leq 0.1$) increased root surface area compared to control.



SFigure 4. Average root diameter of in vitro inoculated Arabidopsis. *Bacillus pumulis* and *Mitsuria* sp. significantly ($\alpha \leq 0.1$) increased average root diameter compared to the un-treated control.



SFigure 5. Root volume of inoculated Arabidopsis in vitro. *Bacillus pumulis*, and *Mitsuria* sp. significantly ($\alpha \leq 0.1$) increased root volume compared to the un-inoculated control.